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(71) Applicant *(for all designated States except US)*: **COLD SPRING HARBOR LABORATORY** [US/US]; Nichols Building, One Bungtown Road, Cold Spring Harbor, NY 11724 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **TONKS, Nicholas, K.** [GB/US]; 3 Arrowhead Place, Huntington, NY 11732 (US). **ZHANG, Shao-Hui** [US/US]; 13415 Bavarian Drive, San Diego, CA 92129 (US).

(74) Agents: **CARROLL, Alice, O.** et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

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(54) Title: SUBSTRATE TRAPPING PROTEIN TYROSINE PHOSPHATASES

(57) Abstract: Compositions and methods are provided pertaining to novel substrate trapping mutant protein tyrosine phosphatases (PTPs) that are catalytically impaired but which retain the ability to bind phosphotyrosine-containing protein substrate(s), and that are further modified by the replacement of at least one tyrosine residue with an amino acid that cannot be phophorylated. Uses of such PTPs for identification of PTP substrates, and of agents that alter PTP-substrate interactions are disclosed, as are methods of altering PTP activities.

SUBSTRATE TRAPPING PROTEIN TYROSINE PHOSPHATASES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a Continuation-in-Part of U.S. Application Serial No. 09/144,925, filed September 1, 1998, which is a Divisional of U.S. Application No. 08/685,992, filed July 25, 1996. This application also claims the benefit of U.S. Provisional Application No. 60/137,319 (attorney docket number CSHL99-06P), filed June 3, 1999. The teachings of these applications are incorporated herein by reference in their entirety

STATEMENT OF GOVERNMENT INTEREST

Work described herein was supported by government funding under research grant CA 53840 awarded by the National Institutes of Health. The government may have certain rights in this invention.

TECHNICAL FIELD

The present invention relates generally to compositions and methods useful for treating conditions associated with defects in cellular biochemical pathways such as those controlling cell proliferation, cell differentiation and/or cell survival. The invention is more particularly related to substrate trapping mutants of protein tyrosine phosphatase polypeptides, and variants thereof. The present invention is also related to the use of such polypeptides to identify antibodies and other agents, including small molecules, that modulate biological signal transduction and cellular biochemical pathways.

BACKGROUND OF THE INVENTION

Reversible protein tyrosine phosphorylation, coordinated by the action of protein tyrosine kinases (PTKs) that phosphorylate certain tyrosine residues in polypeptides, and protein tyrosine phosphatases (PTPs) that dephosphorylate certain phosphotyrosine residues, is a key mechanism in regulating many cellular activities. It is becoming apparent that the diversity and complexity of the PTPs and PTKs are comparable, and that PTPs are equally important in delivering both positive and negative signals for proper function of cellular machinery. Regulated tyrosine phosphorylation contributes to specific pathways for biological signal transduction, including those associated with cell division, proliferation and differentiation. Defects and/or malfunctions in these pathways may underlie certain disease conditions for which effective means for intervention remain elusive, including for example, malignancy, autoimmune disorders, diabetes, obesity and infection.

The protein tyrosine phosphatase (PTP) family of enzymes consists of more than 500 structurally diverse proteins that have in common the highly conserved 250 amino acid PTP catalytic domain, but which display considerable variation in their non-catalytic segments (Charbonneau and Tonks, 1992 *Annu. Rev. Cell Biol. 8*:463-493; Tonks, 1993 *Semin. Cell Biol. 4*:373-453). This structural diversity presumably reflects the diversity of physiological roles of individual PTP family members, which in certain cases have been demonstrated to have specific functions in growth, development and differentiation (Desai et al., 1996 *Cell 84*:599-609; Kishihara et al., 1993 *Cell 74*:143-156; Perkins et al., 1992 *Cell 70*:225-236; Pingel and Thomas, 1989 *Cell 58*:1055-1065; Schultz et al..1993 *Cell 73*:1445-1454).

Although recent studies have also generated considerable information regarding the structure, expression and regulation of PTPs, the nature of the tyrosine phosphorylated substrates through which the PTPs exert their effects remains to be determined. Studies with a limited number of synthetic phosphopeptide substrates have demonstrated some differences in the substrate selectivities of different PTPs (Cho et al., 1993 *Protein Sci. 2*: 977-984; Dechert et al., 1995 *Eur. J. Biochem. 231*:673-681). Analyses of PTP-mediated dephosphorylation of PTP substrates suggest that catalytic

activity may be favored by the presence of certain amino acid residues at specific positions in the substrate polypeptide relative to the phosphorylated tyrosine residue (Ruzzene et al., 1993 *Eur. J. Biochem. 211*:289-295; Zhang et al., 1994 *Biochemistry 33*:2285-2290). Thus, although the physiological relevance of the substrates used in these studies is unclear, PTPs display a certain level of substrate selectivity *in vitro*.

The PTP family of enzymes contains a common evolutionarily conserved segment of approximately 250 amino acids known as the PTP catalytic domain. Within this conserved domain is a unique signature sequence motif,

[I/V]HCXAGXXR[S/T]G SEQ ID NO:36,

that is invariant among all PTPs. The cysteine residue in this motif is invariant in members of the family and is known to be essential for catalysis of the phosphotyrosine dephosphorylation reaction. It functions as a nucleophile to attack the phosphate moiety present on a phosphotyrosine residue of the incoming substrate. If the cysteine residue is altered by site-directed mutagenesis to serine (*e.g.*, in cysteine-to-serine or "CS" mutants) or alanine (*e.g.*, cysteine-to-alanine or "CA" mutants), the resulting PTP is catalytically attenuated but retains the ability to complex with, or bind, its substrate, at least *in vitro*. Such mutants can be made, for example, using the PTP family member MKP-1 (Sun et al., 1993 *Cell 75*:487–493), as well as other PTPs. However, although these CS mutants can in general bind effectively to phosphotyrosyl substrates *in vitro* to form stable enzyme-substrate complexes, in many cases such complexes cannot be isolated *in vivo*, for example when both the mutant PTP and the phosphotyrosyl protein substrate are present together within a cell. Thus, the CS mutants are of limited usefulness and cannot be employed to isolate all combinations of PTPs and substrates.

Currently, desirable goals for determining the molecular mechanisms that govern PTP-mediated cellular events include, *inter alia*, determination of PTP interacting molecules, substrates and binding partners, and identification of agents that regulate PTP activities. In some situations, however, current approaches may lead to an understanding of certain aspects of the regulation of tyrosine phosphorylation by PTPs, but still may not provide strategies to control specific tyrosine phosphorylation and/or dephosphorylation events within a cell.

Accordingly, there is a need in the art for an improved ability to regulate phosphotyrosine signaling, including regulation of PTPs. An increased understanding of PTP regulation may facilitate the development of methods for modulating the activity of proteins involved in phosphotyrosine signaling pathways, and for treating conditions associated with such pathways. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

The present invention provides novel substrate trapping mutant or altered forms of mammalian PTPs, also referred to as substrate trapping PTPs (ST-PTPs), which bind (trap) one or more substrates of the PTP. Binding of the ST-PTP to a PTP substrate results in the formation of a complex that can be readily observed, and, if desired, isolated, and characterized. These mutant forms of PTPs have attenuated catalytic activity (lack catalytic activity or have reduced catalytic activity) relative to the wild type PTP, but retain the ability to bind tyrosine phosphorylated substrate(s) of the wild type PTP. ST-PTPs are useful, for example, to determine the fine substrate specificity of one or more PTPs.

It is an aspect of the invention to provide a substrate trapping mutant protein tyrosine phosphatase in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In certain embodiments at least one wildtype tyrosine residue is replaced with an amino acid that is alanine, cysteine, aspartic acid, glutamine, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, arginine, valine or tryptophan. In certain other embodiments at least one tyrosine residue that is replaced is located in a protein tyrosine phosphatase catalytic domain. In certain embodiments at least one tyrosine residue that is replaced is located in a protein tyrosine phosphatase active site, and in certain other embodiments at least one tyrosine residue is replaced

with phenylalanine. In certain other embodiments at least one tyrosine residue that is replaced is a protein tyrosine phosphatase conserved residue, and in certain further embodiments the conserved residue corresponds to tyrosine at amino acid position 676 in human PTPH1. In certain embodiments at least one tyrosine residue is replaced with an amino acid that stabilizes a complex comprising the protein tyrosine phosphatase and at least one substrate molecule. In certain embodiments the substrate trapping mutant comprises a mutated PTPH1, and in certain embodiments the substrate trapping mutant comprises a mutated protein tyrosine phosphatase that is PTP1B, PTP-PEST, PTPγ, MKP-1, DEP-1, PTPμ, PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR or PTPH1. In certain embodiments the substrate trapping mutant comprises a mutated PTP-PEST phosphatase in which the amino acid at position 231 is replaced with a serine residue.

It is another aspect of the present invention to provide a method of identifying a tyrosine phosphorylated protein which is a substrate of a protein tyrosine phosphatase, comprising the steps of combining a sample comprising at least one tyrosine phosphorylated protein with at least one substrate trapping mutant protein tyrosine phosphatase, in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, under conditions and for a time sufficient to permit formation of a complex between the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase; and determining the presence or absence of a complex comprising the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase, wherein the presence of the complex indicates that the tyrosine phosphorylated protein is a substrate of the protein tyrosine phosphatase with which it forms a complex. In certain embodiments the substrate trapping mutant comprises a mutated protein tyrosine phosphatase that is PTP1B, PTP-PEST, PTPγ, MKP-1, DEP-1, PTPμ, PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR or PTPH1. In certain embodiments the

sample comprises a cell that expresses the tyrosine phosphorylated protein. and in certain further embodiments the cell has been transfected with at least one nucleic acid molecule encoding the substrate. In certain other embodiments at least one substrate trapping mutant protein tyrosine phosphatase is expressed by a cell. and in certain further embodiments the cell has been transfected with at least one nucleic acid molecule encoding the substrate trapping mutant protein tyrosine phosphatase. In certain other embodiments the sample comprises a cell that expresses (i) the tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase, and (ii) the substrate trapping mutant protein tyrosine phosphatase. In certain other embodiments the cell has been transfected with (i) at least one nucleic acid encoding the substrate. and (ii) at least one nucleic acid encoding the substrate trapping mutant protein tyrosine phosphatase. In certain other embodiments the sample comprises a cell lysate containing at least one tyrosine phosphorylated protein. and in certain further embodiments the cell lysate is derived from a cell transfected with at least one nucleic acid encoding the tyrosine phosphorylated protein. In certain other further embodiments the cell lysate is derived from a cell transfected with at least one nucleic acid encoding a protein tyrosine kinase. In certain other embodiments at least one substrate trapping mutant protein tyrosine phosphatase is present within a cell lysate. and in certain further embodiments the cell lysate is derived from a cell transfected with at least one nucleic acid encoding the substrate trapping mutant protein tyrosine phosphatase. In other embodiments. the tyrosine phosphorylated protein is VCP. $p130^{cas}$, the EGF receptor. p210 bcr:abl. MAP kinase. Shc (Tiganis et al., 1998 *Mol. Cell. Biol.* 18:1622-1634) or the insulin receptor.

Turning to another aspect. the present invention provides a method of identifying an agent which alters the interaction between a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase. comprising contacting in the absence and in the presence of a candidate agent. a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase under conditions and for a time sufficient for detectable dephosphorylation of the substrate to occur. wherein the tyrosine

phosphorylated protein which is a substrate of the protein tyrosine phosphatase is identified by (1) combining a sample comprising at least one tyrosine phosphorylated protein with at least one substrate trapping mutant protein tyrosine phosphatase, in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, under conditions and for a time sufficient to permit formation of a complex between the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase; and (2) determining the presence or absence of a complex comprising the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase, wherein the presence of the complex indicates that the tyrosine phosphorylated protein is a substrate of the protein tyrosine phosphatase with which it forms a complex; and comparing the level of dephosphorylation of the substrate in the absence of the agent to the level of dephosphorylation of the substrate in the presence of the agent, wherein a difference in the level of substrate dephosphorylation indicates the agent alters the interaction between the protein tyrosine phosphatase and the substrate.

In another aspect, the present invention provides a method of identifying an agent which alters the interaction between a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase, comprising contacting in the absence and in the presence of a candidate agent, a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase under conditions and for a time sufficient to permit formation of a complex between the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase, wherein the substrate trapping mutant protein tyrosine phosphatase comprises a mutated protein tyrosine phosphatase in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute,

and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated; and comparing the level of complex formation in the absence of the agent to the level of complex formation in the presence of the agent, wherein a difference in the level of complex formation indicates the agent alters the interaction between the protein tyrosine phosphatase and the substrate.

In another aspect the invention provides a method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a subject a substrate trapping mutant of a protein tyrosine phosphatase in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, whereby interaction of the substrate trapping mutant protein tyrosine phosphatase with the tyrosine phosphorylated protein reduces the activity of the tyrosine phosphorylated protein. In certain embodiments the tyrosine phosphorylated protein is VCP, $p130^{cas}$, the EGF receptor, p210 bcr:abl, MAP kinase, Shc (Tiganis et al., 1998 *Mol. Cell. Biol.* 18:1622-1634) or the insulin receptor. In certain other embodiments the protein tyrosine phosphatase is PTP1B, PTP-PEST, PTPγ, MKP-1, DEP-1, PTPμ, PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR or PTPH1.

In still another aspect the invention provides a method of reducing a transforming effect of at least one oncogene associated with $p130^{cas}$ phosphorylation comprising administering to a mammal capable of expressing $p130^{cas}$ a substrate trapping mutant of PTP-PEST in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated; whereby the substrate trapping mutant interacts with $p130^{cas}$ to reduce the transforming effect of at

least one oncogene associated with $p130^{cas}$ phosphorylation. In certain embodiments the oncogene is v-crk, v-src or c-Ha-ras.

Turning to another aspect, the present invention provides a method of reducing formation of signaling complexes associated with $p130^{cas}$, comprising administering to a mammal capable of expressing $p130^{cas}$ a substrate trapping mutant of PTP-PEST in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated; whereby the substrate trapping mutant interacts with $p130^{cas}$ to reduce the formation of signaling complexes associated with $p130^{cas}$.

The present invention provides, in another aspect, a method of reducing cytotoxic effects associated with protein tyrosine phosphatase administration or overexpression, comprising administering to a mammal a substrate trapping mutant of a protein tyrosine phosphatase in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

Turning now to another aspect of the invention, there is provided an isolated nucleic acid molecule encoding a substrate trapping mutant protein tyrosine phosphatase in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and in which at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In certain embodiments the invention provides an antisense oligonucleotide comprising at least 15 consecutive nucleotides complementary to the nucleic acid molecule encoding a substrate trapping mutant protein tyrosine phosphatase, as just described.

It is another aspect of the invention to provide a fusion protein comprising a polypeptide sequence fused to a substrate trapping mutant protein tyrosine phosphatase in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and in which at least one wildtype protein tyrosine phosphatase tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In certain embodiments the polypeptide is an enzyme or a variant or fragment thereof. In some embodiments the polypeptide sequence fused to a substrate trapping mutant protein tyrosine phosphatase is cleavable by a protease. In certain other embodiments the polypeptide sequence is an affinity tag polypeptide having affinity for a ligand.

In still another aspect, the present invention provides a recombinant expression construct comprising at least one promoter operably linked to a nucleic acid encoding a substrate trapping mutant protein tyrosine phosphatase in which wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and in which at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In certain embodiments the promoter is a regulated promoter, and in certain other embodiments the substrate trapping mutant protein tyrosine phosphatase is expressed as a fusion protein with a polypeptide product of a second nucleic acid sequence. In certain further embodiments the polypeptide product of the second nucleic acid sequence is an enzyme. In certain other embodiments the expression construct is a recombinant viral expression construct. In certain other embodiments the present invention provides a host cell comprising a recombinant expression construct according to those just described. In certain embodiments the host cell is a prokaryotic cell and in certain embodiments the host cell is a eukaryotic cell.

The present invention provides, in another aspect, a method of producing a recombinant substrate trapping mutant protein tyrosine phosphatase, comprising

culturing a host cell comprising a recombinant expression construct comprising at least one promoter operably linked to a nucleic acid sequence encoding a substrate trapping mutant protein tyrosine phosphatase in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and in which at least one wildtype protein tyrosine phosphatase tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In certain embodiments the promoter is a regulated promoter. In certain other embodiments the invention provides a method of producing a recombinant substrate trapping mutant protein tyrosine phosphatase, comprising culturing a host cell infected with the recombinant viral expression construct described above.

The present invention, in another aspect, provides a pharmaceutical composition comprising a substrate trapping mutant protein tyrosine phosphatase in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and in which at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, in combination with a pharmaceutically acceptable carrier or diluent.

In yet another aspect the invention provides a pharmaceutical composition comprising an agent that interacts with a substrate trapping mutant protein tyrosine phosphatase in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and in which at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, in combination with a pharmaceutically acceptable carrier or diluent. In certain other embodiments the invention provides a kit for identifying a tyrosine phosphorylated protein substrate of a protein tyrosine phosphatase comprising at least one substrate trapping mutant protein tyrosine phosphatase in which (i) the wildtype protein tyrosine phosphatase catalytic

domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated; and ancillary reagents suitable for use in detecting the presence or absence of a complex between the protein tyrosine phosphatase and a tyrosine phosphorylated protein.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references (including websites) disclosed herein are hereby incorporated by reference in their entireties as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E show a multiple amino acid sequence alignment of the catalytic domains of various PTPs. The positions of amino acid residues of PTP1B that interact with substrate are indicated with small arrowheads, and the residue numbering at the bottom of the alignment corresponds to that for PTP1B. Figs. 1A-1E show a multiple sequence alignment of the catalytic domains of PTPs (SEQ ID NOS:1-35). Cytosolic eukaryotic PTPs and domain 1 of RPTPs are combined into one group: domains 2 of RPTPs are in a second group and the *Yersinia* PTP is in a third. Invariant residues shared among all three groups are shown in lower case. Invariant and highly conserved residues within a group are shown in italics and bold, respectively. Within the *Yersinia* PTP sequence, residues that are either invariant or highly conserved between the cytosolic and RPTP domain sequences are in italics and bold, respectively.

Figure 2 shows the Vmax, Kcat and Km of various PTP1B mutants toward RCML (reduced and carboxyamidomethylated and maleylated lysozyme).

Figure 3 presents phase contrast micrographs that show growth inhibition of stable NIH3T3 cell lines overexpressing PTPH1 (–, induced; -, uninduced).

Figure 4 presents growth curves (mean values from triplicate plating) that show growth inhibition of stable NIH3T3 cell lines overexpressing PTPH1.

Figure 5 shows inhibition of cell cycle progression by PTPH1 overexpression at indicated time after release from hydroxyurea block by immunoblot analysis using antibodies specific for HA epitope tag (PTPH1) or cyclin (+, induced; -, uninduced).

Figure 6 shows identification of pp97/VCP as a PTPH1 substrate *in vitro* by anti-phosphotyrosine immunoblot analysis of 293 cell lysate proteins trapped by substrate trapping mutant PTPH1(D811A).

Figure 7 shows the amino acid sequence of pp97/VCP (ncbi database accession number Z14044) [SEQ ID NO:42].

Figure 8 shows identification of pp97/VCP as a PTPH1 substrate in vivo by immunoblot analysis of 293 cellular proteins trapped by and co-immunoprecipitated with substrate trapping mutant PTPH1(Y676F/D811A).

Figure 9 shows localization of VCP tyrosine residues recognized by PTPH1 to the C-terminal region of VCP.

Figure 10 shows dephosphorylation of VCP in stable NIH3T3 cell lines expressing wildtype PTPH1.

Figure 11 shows overall profile of tyrosine phosphorylated proteins in stable NIH3T3 cell lines expressing wildtype PTPH1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to novel substrate trapping mutant protein tyrosine phosphatases (PTPs) derived from a PTP that has been mutated such that the PTP catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Michaelis-Menten constant (Km) of the enzyme but which results in a reduction of the catalytic rate constant (Kcat), and that has further been mutated by replacement of at least one tyrosine residue with an amino acid that is not capable of being phosphorylated. The invention is based, in part, on the unexpected finding that under certain conditions *in vivo*, a PTP enzyme may itself undergo tyrosine phosphorylation in a manner that can alter interactions between the PTP and other molecules, including PTP substrates. As defined herein, a

phosphatase is a member of the PTP family if it contains the signature motif [I/V]HCXAGXXR[S/T]G (SEQ ID NO:36). Dual specificity PTPs, *i.e.*, PTPs which dephosphorylate both phosphorylated tyrosine and phosphorylated serine or threonine, are also suitable for use in the invention. Appropriate PTPs include, but are not limited to, PTP1B, PTP-PEST, PTPγ, MKP-1, DEP-1, PTPμ, PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR and PTPH1.

As noted above, substrate trapping mutant PTPs are derived from wildtype PTPs that have been mutated such that the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In this regard, amino acid sequence analysis of known PTPs reveals the presence of twenty seven invariant residues within the PTP primary structure (Barford et al., 1994 *Science 263*:1397-1404; Jia et al., 1995 *Science 268*:1754-1758), including an aspartate residue in the catalytic domain that is invariant among PTP family members. When the amino acid sequences of multiple PTP family members are aligned (see, for instance, Figure 1A-E; see also, *e.g.*, Barford et al., 1995 *Nature Struct. Biol.* 2:1043), this invariant aspartate residue may be readily identified in the catalytic domain region of each PTP sequence at a corresponding position relative to the PTP signature sequence motif [I/V]HCXAGXXR[S/T]G (SEQ ID NO:36), which is invariant among all PTPs (see, *e.g.*, WO98/04712; Flint et al., 1997 *Proc. Nat. Acad. Sci.* 94:1680 and references cited therein). However, the exact amino acid sequence position numbers of catalytic domain invariant aspartate residues may be different from one PTP to another, due to sequence shifts that may be imposed to maximize alignment of the various PTP sequences (see, *e.g.*, Barford et al., 1995 *Nature Struct. Biol.* 2:1043 for an alignment of various PTP sequences).

In particular, portions of two PTP polypeptide sequences are regarded as "corresponding" amino acid sequences, regions, fragments or the like, based on a convention of numbering one PTP sequence according to amino acid position number.

and then aligning the sequence to be compared in a manner that maximizes the number of amino acids that match or that are conserved residues, for example, that remain polar (*e.g.*, D, E, K, R, H, S, T, N, Q), hydrophobic (*e.g.*, A, P, V, L, I, M, F, W, Y) or neutral (e.g., C, G) residues at each position. Similarly, a DNA sequence encoding a candidate PTP that is to be mutated as provided herein, or a portion, region, fragment or the like, may correspond to a known wildtype PTP-encoding DNA sequence according to a convention for numbering nucleic acid sequence positions in the known wildtype PTP DNA sequence, whereby the candidate PTP DNA sequence is aligned with the known PTP DNA such that at least 70%, preferably at least 80% and more preferably at least 90% of the nucleotides in a given sequence of at least 20 consecutive nucleotides of a sequence are identical. In certain preferred embodiments, a candidate PTP DNA sequence is greater than 95% identical to a corresponding known PTP DNA sequence. In certain particularly preferred embodiments, a portion, region or fragment of a candidate PTP DNA sequence is identical to a corresponding known PTP DNA sequence. As is well known in the art, an individual whose DNA contains no irregularities (*e.g.*, a common or prevalent form) in a particular gene responsible for a given trait may be said to possess a wildtype genetic complement (genotype) for that gene, while the presence of irregularities known as mutations in the DNA for the gene, for example, substitutions, insertions or deletions of one or more nucleotides, indicates a mutated or mutant genotype.

As noted above, in certain embodiments of the present invention there is provided a substrate trapping mutant PTP in which catalytic domain invariant aspartate and at least one tyrosine residue are replaced, as provided herein. Identification of the catalytic domain invariant aspartate residue in PTP sequences other than those disclosed in Barford et al. (1995) may be achieved by comparing sequences using computer algorithms well known to those having ordinary skill in the art, such as GENEWORKS. Align or the BLAST algorithm (Altschul, *J. Mol. Biol. 219*:555-565, 1991; Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA 89*:10915-10919, 1992), which is available at the NCBI website (http://www/ncbi.nlm.nih.gov/cgi-bin/BLAST).

Certain embodiments of the invention pertain in part to novel PTPs in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}). These PTPs retain the ability to form a complex with, or bind to, their tyrosine phosphorylated substrates, but are catalytically attenuated (*i.e.*, a substrate trapping mutant PTP retains a similar Km to that of the corresponding wildtype PTP, but has a Vmax which is reduced by a factor of at least 10^2-10^5 relative to the wildtype enzyme, depending on the activity of the wildtype enzyme relative to a Kcat of less than 1 min^{-1}). This attenuation includes catalytic activity which is either reduced or abolished relative to the wildtype PTP. For example, the invariant aspartate residue can be changed or mutated to an alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, lysine, arginine or histidine.

The preferred substrate trapping mutant PTPs described herein, in which the invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}), and in which at least one tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, may further comprise other mutations. In particularly preferred embodiments, such additional mutations relate to substitutions, insertions or deletions (most preferably substitutions) that assist in stabilizing the PTP/substrate complex. For example, mutation of the serine/threonine residue in the signature motif to an alanine residue (S/T→A mutant) may change the rate-determining step of the PTP-mediated substrate dephosphorylation reaction. For the unmodified PTP, formation of the transition state may be rate-limiting, whereas in the case of the S/T→A mutant, the breakdown of the transition state may become rate-limiting, thereby stabilizing the PTP/substrate complex. Such mutations may be valuably combined with the replacement of the PTP catalytic domain invariant aspartate residue and the replacement of PTP tyrosine as provided herein, for example, with regard to stabilizing the PTP-substrate complex and facilitating its isolation. As another example, substitution of any one or more other amino acids

present in the wildtype PTP that are capable of being phosphorylated as provided herein (*e.g.*, serine, threonine, tyrosine) with an amino acid that is not capable of being phosphorylated may be desirable, with regard to the stability of a PTP-substrate complex.

As noted above, the present invention provides substrate trapping mutant PTPs in which catalytic domain invariant aspartate and at least one tyrosine residue are replaced, wherein the tyrosine is replaced with an amino acid that is not capable of being phosphorylated. The amino acid that is not capable of being phosphorylated may, in preferred embodiments, be alanine, cysteine, aspartic acid, glutamine, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, arginine, valine or tryptophan. The desirability of the tyrosine replacement derives from the surprising observation that under certain conditions *in vivo*, a PTP enzyme may itself undergo tyrosine phosphorylation in a manner that can alter interactions between the PTP and other molecules, including PTP substrates. PTP substrates include any naturally or non-naturally tyrosine-phosphorylated peptide, polypeptide or protein that can specifically bind to and/or be dephosphorylated by a PTP as provided herein. Thus, replacement of a tyrosine residue found in the wildtype amino acid sequence of a particular PTP with another amino acid as provided herein stabilizes a complex formed by the subject invention substrate trapping mutant PTP and a PTP substrate when the amount of complex that is present and/or the affinity of the mutant PTP for the substrate increases, relative to complex formation using a PTP in which the tyrosine residue is not replaced.

As noted above, the present invention exploits the substrate trapping mutant PTPs described herein to provide a method of identifying a tyrosine phosphorylated protein that is a substrate of a wildtype PTP. According to this aspect of the invention, a sample comprising at least one tyrosine phosphorylated protein is combined with at least one substrate trapping mutant PTP as provided herein, and the presence or absence of a complex comprising the substrate and the mutant PTP is determined. The binding interaction between a PTP and a PTP substrate may result in the formation of a complex, which refers to the affinity interaction of the PTP and the

PTP substrate. A complex may include a signaling complex, which refers to any complex that, by virtue of its formation, its stable association and/or its dissociation directly or indirectly provides a biological signal. Such signals may include, for example by way of illustration and not limitation, intracellular and/or intercellular events that lead to molecular binding, covalent or non-covalent modification of molecular structure, gene expression, genetic recombination, genetic integration, nucleic acid synthesis or subcellular particle assembly, and may also include endocytic, phagocytic, nucleolytic, proteolytic, lipolytic, hydrolytic, catalytic, or other regulatory events.

Determination of the presence of a stable complex between a PTP and a PTP substrate refers to the use of any methodology known in the art for demonstrating an intermolecular interaction between a PTP and a PTP substrate according to the present disclosure. Such methodologies may include, by way of illustration and not limitation, co-purification, co-precipitation, co-immunoprecipitation, radiometric or fluorimetric assays, western immunoblot analyses, affinity capture including affinity techniques such as solid-phase ligand-counterligand sorbent techniques, affinity chromatography and surface affinity plasmon resonance, and the like. For these and other useful affinity techniques, see, for example, Scopes, R.K., *Protein Purification: Principles and Practice*, 1987, Springer-Verlag, NY; Weir, D.M., *Handbook of Experimental Immunology*, 1986, Blackwell Scientific, Boston; and Hermanson, G.T. et al., *Immobilized Affinity Ligand Techniques*, 1992, Academic Press, Inc., California; which are hereby incorporated by reference in their entireties, for details regarding techniques for isolating and characterizing complexes, including affinity techniques. A PTP may interact with a PTP substrate via specific binding if the PTP binds the substrate with a K_a of greater than or equal to about 10^4 M^{-1}, preferably of greater than or equal to about 10^5 M^{-1}, more preferably of greater than or equal to about 10^6 M^{-1} and still more preferably of greater than or equal to about 10^7 M^{-1} to 10^9 M^{-1}. Affinities of binding partners such as a PTP and a PTP substrate can be readily determined using conventional techniques, for example those described by Scatchard et al., *Ann. N.Y. Acad. Sci. 51*:660 (1949).

Without wishing to be bound by theory, it is contemplated that phosphorylated tyrosine residues that are part of a PTP molecule itself may influence the interaction between the PTP molecule and PTP substrate molecules, which include tyrosine phosphorylated proteins that a PTP may bind and/or dephosphorylate. According to this non-limiting theory, a conserved tyrosine residue present in a PTP primary structure may be a receptor for transfer of a phosphate group from the highly reactive thiophosphate intermediate that may be formed between the invariant cysteine residue found in the signature motif that resides in the active site of the PTP catalytic domain (as described above) and the phosphate group present in the form of phosphotyrosine on the PTP substrate phosphoprotein. Thus, although a conserved tyrosine residue in a PTP active site may facilitate intermolecular orientation of the PTP relative to its substrate by providing a hydrophobic interaction with the substrate phosphotyrosine, and may further act as a phosphate acceptor, the invention is not so limited.

As described above, the present invention provides a mutated PTP in which at least one tyrosine residue is replaced with an amino acid that cannot be phosphorylated. Preferably the tyrosine residue is located in the PTP catalytic domain, which refers to the approximately 250 amino acid region that is highly conserved among the various PTPs, as noted above (see also, *e.g.*, Barford, 1998 *Ann. Rev. Biophys. Biomol. Struct.* 27:133; Jia, 1997 *Biochem. Cell Biol.* 75:17; Van Vactor et al., 1998 *Curr. Opin Genet. Devel.* 8:112) More preferably, the tyrosine residue is located in a PTP active site, which refers to the region within the PTP catalytic domain that contains the PTP signature motif and which also includes those amino acids that form the PTP binding site pocket or "cradle" for substrate binding and dephosphorylation, further including the invariant aspartate-containing loop (when present) and adjacent peptide backbone sequences that contribute to substrate recognition and catalysis (see, *e.g.*, Jia, 1997). In a most preferred embodiment, the tyrosine residue is replaced with phenylalanine, and in another most preferred embodiment, the tyrosine residue is a conserved residue that corresponds to the tyrosine situated at position 676 in the amino acid sequence of human PTPH1, and which also corresponds to the amino acid residue

at position 46 in the PTP-1B sequence shown in Figure 1. In other preferred embodiments, the tyrosine residue is a PTP conserved residue, which includes tyrosine residues that are present at corresponding positions within two or more PTP amino acid sequences relative to the position of the signature motif. In other preferred embodiments, the tyrosine residue is replaced with an amino acid that stabilizes a complex formed by the PTP and at least one substrate molecule, as provided herein.

As noted above, PTPs that may be useful according to the present invention include any PTP which has an invariant aspartate residue in a corresponding position in the catalytic domain, and a tyrosine residue. By way of illustration and not limitation, in certain preferred embodiments of the present invention, the substrate trapping mutant PTP has at least one tyrosine residue found in the corresponding wildtype sequence replaced with phenylalanine. In certain particularly preferred embodiments, the PTP is PTPH1 having the invariant aspartate replaced by alanine and the tyrosine at position 676 replaced by phenylalanine, PTPH1(Y676F/D811A). In certain other embodiments, the PTP is a mutated PTP-PEST phosphatase in which the cysteine found in the corresponding wildtype sequence is replaced with serine and at least one wildtype tyrosine residue is replaced with an amino acid that cannot be phosphorylated. It should be recognized, however, that mutant PTPs other than those specifically described herein can readily be made by aligning the amino acid sequence of a PTP catalytic domain with the amino acid sequence of PTPs that are described herein (including those provided by the cited references), identifying the catalytic domain invariant aspartate residue and at least one tyrosine residue, and changing these residues, for example by site-directed mutagenesis of DNA encoding the PTP.

Modification of DNA may be performed by a variety of methods, including site-specific or site-directed mutagenesis of DNA encoding the PTP and the use of DNA amplification methods using primers to introduce and amplify alterations in the DNA template, such as PCR splicing by overlap extension (SOE). Site-directed mutagenesis is typically effected using a phage vector that has single- and double-stranded forms, such as M13 phage vectors, which are well-known and commercially available. Other suitable vectors that contain a single-stranded phage origin of

replication may be used (*see, e.g.*, Veira et al., *Meth. Enzymol. 15*:3, 1987). In general, site-directed mutagenesis is performed by preparing a single-stranded vector that encodes the protein of interest (*e.g.*, a member of the PTP family). An oligonucleotide primer that contains the desired mutation within a region of homology to the DNA in the single-stranded vector is annealed to the vector followed by addition of a DNA polymerase, such as *E. coli* DNA polymerase I (Klenow fragment), which uses the double stranded region as a primer to produce a heteroduplex in which one strand encodes the altered sequence and the other the original sequence. Additional disclosure relating to site-directed mutagenesis may be found, for example, in Kunkel et al. (*Methods in Enzymol. 154*:367, 1987); and in U.S. Patent Nos. 4,518,584 and 4,737,462. The heteroduplex is introduced into appropriate bacterial cells, and clones that include the desired mutation are selected. The resulting altered DNA molecules may be expressed recombinantly in appropriate host cells to produce the modified protein.

Specific substitutions of individual amino acids through introduction of site-directed mutations are well-known and may be made according to methodologies with which those having ordinary skill in the art will be familiar. The effects on catalytic activity of the resulting mutant PTP may be determined empirically merely by testing the resulting modified protein for the preservation of the Km and reduction of Kcat to less than 1 per minute as provided herein and as previously disclosed (*e.g.*, WO98/04712; Flint et al., 1997 *Proc. Nat. Acad. Sci.* 94:1680). The effects on the ability to tyrosine phosphorylate the resulting mutant PTP molecule can also be determined empirically merely by testing such a mutant for the presence of phosphotyrosine, as also provided herein, for example, following exposure of the mutant to conditions *in vitro* or *in vivo* where it may act as a PTK acceptor.

Although the specific examples of PTP mutants described below are DA (aspartate to alanine) mutants, YF (tyrosine to phenylalanine) mutants, CS mutants and combinations thereof, it will be understood that the subject invention substrate trapping mutant PTPs are not limited to these amino acid substitutions. The invariant aspartate residue can be changed, for example by site-directed mutagenesis, to any amino acid

that does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}). For example, the invariant aspartate residue can be changed or mutated to an alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, lysine, arginine or histidine, or other natural or non-natural amino acids known in the art including derivatives, variants and the like. Similarly, substitution of at least one tyrosine residue may be with any amino acid that is not capable of being phosphorylated (*i.e.*, stable, covalent modification of an amino acid side chain at a hydroxyl with a phosphate group), for example alanine, cysteine, aspartic acid, glutamine, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, arginine, valine or tryptophan, or other natural or non-natural amino acids known in the art including derivatives, variants and the like.

The nucleic acids of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. A nucleic acid molecule encoding a substrate trapping mutant PTP in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and in which at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, may be identical to the coding sequence known in the art for any given PTP, as described above, or may be a different coding sequence, which, as a result of the redundancy or degeneracy of the genetic code, encodes the same PTP.

The present invention further relates to variants of the herein described nucleic acids which encode fragments, analogs and derivatives of a PTP polypeptide, including a mutated PTP such as a substrate trapping mutant PTP. The variants of the nucleic acids encoding PTPs may be naturally occurring allelic variants of the nucleic acids or non-naturally occurring variants. As is known in the art, an allelic variant is an

alternate form of a nucleic acid sequence which may have at least one of a substitution, a deletion or an addition of one or more nucleotides, any of which does not substantially alter the function of the encoded PTP polypeptide.

Equivalent DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity are also encompassed by the invention. For example, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Other equivalents can be prepared by modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites.

The present invention further relates to PTP polypeptides including substrate trapping mutant PTPs, and in particular to methods for producing recombinant PTP polypeptides by culturing host cells containing PTP expression constructs, and to isolated recombinant PTP polypeptides. The polypeptides and nucleic acids of the present invention are preferably provided in an isolated form, and in certain preferred embodiments are purified to homogeneity. The terms "fragment," "derivative" and "analog" when referring to PTP polypeptides or fusion proteins, including substrate trapping mutant PTPs, refers to any PTP polypeptide or fusion protein that retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active PTP polypeptide. The polypeptides of the present

invention may be recombinant polypeptides or synthetic polypeptides, and are preferably recombinant polypeptides.

A fragment, derivative or analog of a PTP polypeptide or fusion protein, including substrate trapping mutant PTPs, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue), and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the PTP polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (*e.g.*, polyethylene glycol), or (iv) one in which additional amino acids are fused to the PTP polypeptide, including amino acids that are employed for purification of the PTP polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides of the present invention include PTP polypeptides and fusion proteins having amino acid sequences that are identical or similar to PTP sequences known in the art. For example by way of illustration and not limitation, the human PTP polypeptides (including substrate trapping mutant PTPs) referred to below in the Examples are contemplated for use according to the instant invention, as are polypeptides having at least 70% similarity (preferably 70% identity), more preferably 90% similarity (more preferably 90% identity) and still more preferably 95% similarity (still more preferably 95% identity) to the polypeptides described in references cited herein and in the Examples and to portions of such polypeptides, wherein such portions of a PTP polypeptide generally contain at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and conserved amino acid substitutes thereto of the polypeptide to the sequence of a second polypeptide (*e.g.*, using GENEWORKS, Align or the BLAST algorithm, as described above). Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding

full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the nucleic acids of the present invention may be used to synthesize full-length nucleic acids of the present invention.

The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living animal is not isolated, but the same nucleic acid or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such nucleic acid could be part of a vector and/or such nucleic acid or polypeptide could be part of a composition, and still be isolated in that such vector or composition is not part of the natural environment for the nucleic acid or polypeptide. The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region "leader and trailer" as well as intervening sequences (introns) between individual coding segments (exons).

As described herein, the invention provides a fusion protein comprising a polypeptide fused to a substrate trapping mutant PTP in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and in which at least one wildtype protein tyrosine phosphatase tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. Such PTP fusion proteins are encoded by nucleic acids have the substrate trapping mutant PTP coding sequence fused in frame to an additional coding sequence to provide for expression of a PTP polypeptide sequence fused to an additional functional or non-functional polypeptide sequence that permits, for example by way of illustration and not limitation, detection, isolation and/or purification of the PTP fusion protein. Such PTP fusion proteins may permit detection, isolation and/or purification of the PTP fusion protein by protein-protein affinity, metal affinity or charge affinity-based polypeptide purification, or by specific protease

cleavage of a fusion protein containing a fusion sequence that is cleavable by a protease such that the PTP polypeptide is separable from the fusion protein.

Thus, PTP fusion proteins may comprise affinity tag polypeptide sequences, which refers to polypeptides or peptides added to PTP to facilitate detection and isolation of the PTP via a specific affinity interaction with a ligand. The ligand may be any molecule, receptor, counterreceptor, antibody or the like with which the affinity tag may interact through a specific binding interaction as provided herein. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., (1988 *Bio/Technology* *6*:1204), or the XPRESS™ epitope tag (Invitrogen, Carlsbad, CA). The affinity sequence may be a hexa-histidine tag as supplied, for example, by a pBAD/His (Invitrogen) or a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the affinity sequence may be a hemagglutinin (HA) tag when a mammalian host, *e.g.*, COS-7 cells, is used. The HA tag corresponds to an antibody defined epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984 *Cell 37*:767).

PTP fusion proteins may further comprise immunoglobulin constant region polypeptides added to PTP to facilitate detection, isolation and/or localization of PTP. The immunoglobulin constant region polypeptide preferably is fused to the C-terminus of a PTP polypeptide. General preparation of fusion proteins comprising heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, *e.g.*, by Ashkenazi et al. (*PNAS USA* *88*:10535, 1991) and Byrn et al. (*Nature* *344*:677, 1990). A gene fusion encoding the PTP:Fc fusion protein is inserted into an appropriate expression vector. In certain embodiments of the invention, PTP:Fc fusion proteins may be allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between Fc polypeptides, yielding dimeric PTP fusion proteins.

PTP fusion proteins having specific binding affinities for pre-selected antigens by virtue of fusion polypeptides comprising immunoglobulin V-region domains encoded by DNA sequences linked in-frame to sequences encoding PTP are

also within the scope of the invention, including variants and fragments thereof as provided herein. General strategies for the construction of fusion proteins having immunoglobulin V-region fusion polypeptides are disclosed, for example, in EP 0318554; U.S. 5,132,405; U.S. 5,091,513; and U.S. 5,476,786.

The nucleic acid of the present invention may also encode a fusion protein comprising a PTP polypeptide fused to other polypeptides having desirable affinity properties, for example an enzyme such as glutathione-S-transferase. As another example, PTP fusion proteins may also comprise a PTP polypeptide fused to a *Staphylococcus aureus* protein A polypeptide; protein A encoding nucleic acids and their use in constructing fusion proteins having affinity for immunoglobulin constant regions are disclosed generally, for example, in U.S. Patent 5,100,788. Other useful affinity polypetides for construction of PTP fusion proteins may include streptavidin fusion proteins, as disclosed, for example, in WO 89/03422; U.S. 5,489,528; U.S. 5,672,691; WO 93/24631; U.S. 5,168,049; U.S. 5,272,254 and elsewhere, and avidin fusion proteins (see, *e.g.*, EP 511,747). As provided herein and in the cited references, PTP polypeptide sequences, including substrate trapping mutant PTPs, may be fused to fusion polypeptide sequences that may be full length fusion polypeptides and that may alternatively be variants or fragments thereof.

The present invention also contemplates PTP fusion proteins that contain polypeptide sequences that direct the fusion protein to the cell nucleus, to reside in the lumen of the endoplasmic reticulum (ER), to be secreted from a cell via the classical ER-Golgi secretory pathway (*see, e.g.*, von Heijne, *J. Membrane Biol. 115*:195-201, 1990), to be incorporated into the plasma membrane, to associate with a specific cytoplasmic component including the cytoplasmic domain of a transmembrane cell surface receptor or to be directed to a particular subcellular location by any of a variety of known intracellular protein sorting mechanisms with which those skilled in the art will be familiar (*See. e.g.*, Rothman, *Nature* 372:55-63, 1994, Adrani et al., 1998 *J. Biol. Chem.* 273:10317, and references cited therein.). Accordingly, these and related embodiments are encompassed by the instant compositions and methods directed to

targeting a polypeptide of interest to a predefined intracellular, membrane or extracellular localization.

The present invention also relates to vectors and to constructs that include nucleic acids of the present invention, and in particular to "recombinant expression constructs" that include any nucleic acids encoding PTP polypeptides according to the invention as provided above; to host cells which are genetically engineered with vectors and/or constructs of the invention and to the production of PTP polypeptides and fusion proteins of the invention, or fragments or variants thereof, by recombinant techniques. PTP proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described, for example, by Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor, New York, (1989).

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product.

Useful expression constructs for bacterial use are constructed by inserting into an expression vector a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The construct may comprise one or more

phenotypic selectable markers and an origin of replication to ensure maintenance of the vector construct and, if desirable, to provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice. Any other plasmid or vector may be used as long as they are replicable and viable in the host.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, Wisconsin, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter, if it is a regulated promoter as provided herein, is induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents; such methods are well know to those skilled in the art.

Thus, for example, the nucleic acids of the invention as provided herein may be included in any one of a variety of expression vector constructs as a recombinant expression construct for expressing a PTP polypeptide. Such vectors and constructs include chromosomal, nonchromosomal and synthetic DNA sequences, *e.g.*, derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA, such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may

be used for preparation of a recombinant expression construct as long as it is replicable and viable in the host.

The appropriate DNA sequence(s) may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Standard techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described, for example, in Ausubel et al. (1993 *Current Protocols in Molecular Biology*, Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., Boston, MA); Sambrook et al. (1989 *Molecular Cloning*, Second Ed., Cold Spring Harbor Laboratory, Plainview, NY); Maniatis et al. (1982 Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, NY); and elsewhere.

The DNA sequence in the expression vector is operatively linked to at least one appropriate expression control sequences (*e.g.*, a promoter or a regulated promoter) to direct mRNA synthesis. Representative examples of such expression control sequences include LTR or SV40 promoter, the *E. coli lac* or *trp*, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art, and preparation of certain particularly preferred recombinant expression constructs comprising at least one promoter or regulated promoter operably linked to a nucleic acid encoding a PTP polypeptide is described herein.

As noted above, in certain embodiments the vector may be a viral vector such as a retroviral vector. For example, retroviruses from which the retroviral plasmid

vectors may be derived include. but are not limited to, Moloney Murine Leukemia Virus. spleen necrosis virus. retroviruses such as Rous Sarcoma Virus. Harvey Sarcoma virus. avian leukosis virus. gibbon ape leukemia virus. human immunodeficiency virus. adenovirus. Myeloproliferative Sarcoma Virus. and mammary tumor virus.

The viral vector includes one or more promoters. Suitable promoters which may be employed include. but are not limited to. the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al.. *Biotechniques* 7:980-990 (1989), or any other promoter (*e.g.*, cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III. and β-actin promoters). Other viral promoters which may be employed include. but are not limited to, adenovirus promoters. thymidine kinase (TK) promoters. and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein. and may be from among either regulated promoters or promoters as described above.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include. but are not limited to. the PE501, PA317, ψ-2, ψ-AM. PA12. T19-14X. VT-19-17-H2, ψCRE. ψCRIP. GP+E-86. GP+envAm12. and DAN cell lines as described in Miller, *Human Gene Therapy*, *1*:5-14 (1990). which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include. but are not limited to, electroporation. the use of liposomes. and calcium phosphate precipitation. In one alternative. the retroviral plasmid vector may be encapsulated into a liposome. or coupled to a lipid. and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the PTP polypeptides or fusion proteins. Such retroviral vector particles then may be employed. to transduce eukaryotic cells. either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the PTP polypeptide or fusion protein. Eukaryotic cells which may be transduced include. but are not limited to. embryonic

stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, bronchial epithelial cells and various other culture-adapted cell lines.

As another example of an embodiment of the invention in which a viral vector is used to prepare the recombinant PTP expression construct, in one preferred embodiment, host cells transduced by a recombinant viral construct directing the expression of PTP polypeptides or fusion proteins may produce viral particles containing expressed PTP polypeptides or fusion proteins that are derived from portions of a host cell membrane incorporated by the viral particles during viral budding. In another preferred embodiment, PTP encoding nucleic acid sequences are cloned into a baculovirus shuttle vector, which is then recombined with a baculovirus to generate a recombinant baculovirus expression construct that is used to infect, for example, Sf9 host cells, as described in *Baculovirus Expression Protocols. Methods in Molecular Biology* Vol. 39, Christopher D. Richardson, Editor, Human Press, Totowa, NJ, 1995; Piwnica-Worms, "Expression of Proteins in Insect Cells Using Baculoviral Vectors," Section II in Chapter 16 in: *Short Protocols in Molecular Biology*, 2nd Ed., Ausubel *et al.*, eds., John Wiley & Sons, New York, New York, 1992, pages 16-32 to 16-48.

In another aspect, the present invention relates to host cells containing the above described recombinant PTP expression constructs. Host cells are genetically engineered (transduced, transformed or transfected) with the vectors and/or expression constructs of this invention which may be, for example, a cloning vector, a shuttle vector or an expression construct. The vector or construct may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying particular genes such as genes encoding PTP polypeptides or PTP fusion proteins. The culture conditions for particular host cells selected for expression, such as temperature, pH and the like, will be readily apparent to the ordinarily skilled artisan.

The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell.

such as a bacterial cell. Representative examples of appropriate host cells according to the present invention include, but need not be limited to, bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells, such as *Drosophila S2* and *Spodoptera Sf9*; animal cells, such as CHO, COS or 293 cells; adenoviruses; plant cells, or any suitable cell already adapted to *in vitro* propagation or so established *de novo*. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

Various mammalian cell culture systems can also be employed to express recombinant protein. The invention is therefore directed in part to a method of producing a recombinant substrate trapping mutant protein tyrosine phosphatase, by culturing a host cell comprising a recombinant expression construct that comprises at least one promoter operably linked to a nucleic acid sequence encoding a substrate trapping mutant protein tyrosine phosphatase in which the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and in which at least one wildtype protein tyrosine phosphatase tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In certain embodiments, the promoter may be a regulated promoter as provided herein, for example a tetracycline-repressible promoter. In certain embodiments the recombinant expression construct is a recombinant viral expression construct as provided herein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell 23*:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences, for example as described herein regarding the preparation of PTP expression constructs. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements. Introduction of the construct into

the host cell can be effected by a variety of methods with which those skilled in the art will be familiar, including but not limited to, for example, calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis et al., 1986 *Basic Methods in Molecular Biology*).

Identification of nucleic acid molecules for use as antisense agents, which includes antisense oligonucleotides and ribozymes specific for nucleic acid sequences encoding PTPs (including substrate trapping mutant PTPs) or variants or fragments thereof; and of DNA oligonucleotides encoding PTP genes (including substrate trapping mutant PTPs) for targeted delivery for genetic therapy, involve methods well known in the art. For example, the desirable properties, lengths and other characteristics of such oligonucleotides are well known. In certain preferred embodiments such an antisense oligonucleotide comprises at least 15 consecutive nucleotides complementary to an isolated nucleic acid molecule encoding a substrate trapping mutant PTP as provided herein. Antisense oligonucleotides are typically designed to resist degradation by endogenous nucleolytic enzymes by using such linkages as: phosphorothioate, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and other such linkages (*see, e.g.*, Agrwal et al., *Tetrehedron Lett. 28*:3539-3542 (1987); Miller et al., *J. Am. Chem. Soc. 93*:6657-6665 (1971); Stec et al., *Tetrehedron Lett. 26*:2191-2194 (1985); Moody et al., *Nucl. Acids Res. 12*:4769-4782 (1989); Uznanski et al., *Nucl. Acids Res.* (1989); Letsinger et al., *Tetrahedron 40*:137-143 (1984); Eckstein, *Annu. Rev. Biochem. 54*:367-402 (1985); Eckstein, *Trends Biol. Sci. 14*:97-100 (1989); Stein In: *Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression*, Cohen, Ed. Macmillan Press, London, pp. 97-117 (1989); Jager et al., *Biochemistry 27*:7237-7246 (1988)).

Antisense nucleotides are oligonucleotides that bind in a sequence-specific manner to nucleic acids, such as mRNA or DNA. When bound to mRNA that has complementary sequences, antisense prevents translation of the mRNA (*see, e.g.*, U.S. Patent No. 5,168,053 to Altman et al.; U.S. Patent No. 5,190,931 to Inouye, U.S. Patent No. 5,135,917 to Burch; U.S. Patent No. 5,087,617 to Smith and Clusel et al. (1993) *Nucl. Acids Res. 21*:3405-3411, which describes dumbbell antisense

oligonucleotides). Triplex molecules refer to single DNA strands that bind duplex DNA forming a colinear triplex molecule, thereby preventing transcription (*see, e.g.*, U.S. Patent No. 5,176,996 to Hogan et al., which describes methods for making synthetic oligonucleotides that bind to target sites on duplex DNA).

According to this embodiment of the invention, particularly useful antisense nucleotides and triplex molecules are molecules that are complementary to or bind the sense strand of DNA or mRNA that encodes a PTP polypeptide (including substrate trapping mutant PTPs), such that inhibition of translation of mRNA encoding the PTP polypeptide is effected.

A ribozyme is an RNA molecule that specifically cleaves RNA substrates, such as mRNA, resulting in specific inhibition or interference with cellular gene expression. There are at least five known classes of ribozymes involved in the cleavage and/or ligation of RNA chains. Ribozymes can be targeted to any RNA transcript and can catalytically cleave such transcripts (*see, e.g.*, U.S. Patent No. 5,272,262; U.S. Patent No. 5,144,019; and U.S. Patent Nos. 5,168,053, 5,180,818, 5,116,742 and 5,093,246 to Cech et al.). According to certain embodiments of the invention, any such PTP (including substrate trapping mutant PTP) mRNA-specific ribozyme, or a nucleic acid encoding such a ribozyme, may be delivered to a host cell to effect inhibition of PTP gene expression. Ribozymes, and the like may therefore be delivered to the host cells by DNA encoding the ribozyme linked to a eukaryotic promoter, such as a eukaryotic viral promoter, such that upon introduction into the nucleus, the ribozyme will be directly transcribed.

The expressed recombinant PTP polypeptides or fusion proteins (including substrate trapping mutant PTPs) may be useful in intact host cells; in intact organelles such as cell membranes, intracellular vesicles or other cellular organelles; or in disrupted cell preparations including but not limited to cell homogenates or lysates, microsomes, uni- and multilamellar membrane vesicles or other preparations. Alternatively, expressed recombinant PTP polypeptides or fusion proteins can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange

chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

Turning to another aspect of the invention, there is provided a method of identifying a tryosine phosphorylated protein which is a substrate of a PTP. A "sample" as used herein refers to a biological sample containing at least one tyrosine phosphorylated protein, and may be provided by obtaining a blood sample, biopsy specimen, tissue explant, organ culture or any other tissue or cell preparation from a subject or a biological source. A sample may further refer to a tissue or cell preparation in which the morphological integrity or physical state has been disrupted, for example, by dissection, dissociation, solubilization, fractionation, homogenization, biochemical or chemical extraction, pulverization, lyophilization, sonication or any other means for processing a sample derived from a subject or biological source. In certain preferred embodiments, the sample is a cell lysate, and in certain particularly preferred embodiments the lysate is a detergent solubilized cell lysate from which insoluble components have been removed according to standard cell biology techniques. The subject or biological source may be a human or non-human animal, a primary cell culture or culture adapted cell line including but not limited to genetically engineered cell lines that may contain chromosomally integrated or episomal recombinant nucleic acid sequences, immortalized or immortalizable cell lines, somatic cell hybrid cell lines, differentiated or differentiatable cell lines, transformed cell lines and the like. Optionally, in certain situations it may be desirable to treat cells in a biological sample with pervanadate as described herein, to enrich the sample in tyrosine phosphorylated proteins. Other means may also be employed to effect an increase in the population of tyrosine phosphorylated proteins present in the sample, including the use of a subject or biological source that is a cell line that has been transfected with at least one gene encoding a protein tyrosine kinases. Additionally or alternatively, protein tyrosine phosphorylation may be stimulated in subject or biological source cells using any one or

more of a variety of well known methods and compositions known in the art to stimulate protein tyrosine kinase activity. These stimuli may include, without limitation, exposure of cells to cytokines, growth factors, hormones, peptides, small molecule mediators or other agents that induce PTK-mediated protein tyrosine phosphorylation. Such agents may include, for example, interleukins, interferons, human growth hormone, insulin and fibroblast growth factor (FGF), as well as other agents with which those having ordinary skill in the art will be familiar.

According to the subject invention, a sample comprising at least one tyrosine phosphorylated protein is combined with at least one substrate trapping mutant PTP as provided herein, under conditions and for a time sufficient to permit formation of a complex between the tyrosine phosphorylated protein and the substrate trapping mutant PTP. Suitable conditions for formation of such complexes are known in the art and can be readily determined based on teachings provided herein, including solution conditions and methods for detecting the presence of a complex. Next, the presence or absence of a complex comprising the tyrosine phosphorylated protein and the substrate trapping mutant PTP is determined, wherein the presence of the complex indicates that the tyrosine phosphorylated protein is a substrate of the PTP with which it forms a complex.

Substrate trapping mutant PTPs that associate in complexes with tyrosine phosphorylated protein substrates may be identified by any of a variety of techniques known in the art for demonstrating an intermolecular interaction between a PTP and a PTP substrate as described above, for example, co-purification, co-precipitation, co-immunoprecipitation, radiometric or fluorimetric assays, western immunoblot analyses, affinity capture including affinity techniques such as solid-phase ligand-counterligand sorbent techniques, affinity chromatography and surface affinity plasmon resonance, and the like (see, *e.g.*, U.S. Patent No. 5,352,660). Determination of the presence of a PTP/substrate complex may employ antibodies, including monoclonal, polyclonal, chimeric and single-chain antibodies, and the like, that specifically bind to the PTP or the tyrosine phosphorylated protein substrate. Labeled PTPs and/or labeled tyrosine phosphorylated substrates can also be used to detect the

presence of a complex. The PTP or phosphorylated protein can be labeled by covalently or non-covalently attaching a suitable reporter molecule or moiety, for example any of various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include, but are not limited to, horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase and acetylcholinesterase. Examples of suitable fluorescent materials include, but are not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin. Appropriate luminescent materials include luminol, and suitable radioactive materials include radioactive phosphorus [^{32}P], iodine [^{125}I or ^{131}I] or tritium [^{3}H].

Using such approaches, representative complexes of PTP1B with p210 bcr:abl, of PTP-PEST with p130^{cas}, of TC-PTP with Shc (*e.g.*, Tiganis et al., 1998 *Mol. Cell. Biol.* 18:1622-1634) and of PTPH1 with pp97/VCP may be readily identified by western immunoblot analysis as described below. These associations may be observed, for example, in lysates from several cell lines and in transfected cells, indicating that p210 bcr:abl, p130^{cas}, Shc and VCP represent major physiologically relevant substrates for PTP1B, PTP-PEST, TC-PTP and PTPH1, respectively. The compositions and methods of the present invention, which may be used, as exemplified herein, to identify specific tyrosine phosphorylated substrates for PTP1B, PTP-PEST and PTPH1, are generally applicable to any member of the PTP family, including but not limited to TC-PTP, PTPγ, MKP-1, DEP-1, PTPμ, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, CD45, LAR and PTPX10.

In certain embodiments of this aspect of the invention, the sample may comprise a cell that naturally expresses the tyrosine phosphorylated protein that is a PTP substrate, while in certain other embodiments the sample may comprise a cell that has been transfected with one or more nucleic acid molecules encoding the substrate protein. For example, the sample may comprise a cell or population of cells that has been transfected with a nucleic acid library such as a cDNA library that contains at least one nucleic acid molecule encoding a substrate protein. Any tyrosine phosphorylated protein is suitable as a potential substrate in the present invention. Tyrosine

phosphorylated proteins are well known in the art. Specific examples of appropriate substrates include, without limitation, $p130^{cas}$, pp97/VCP, the EGF receptor, p210 bcr:abl, MAP kinase, Shc and the insulin receptor. Of particular interest are tyrosine phosphorylated proteins that have been implicated in a mammalian disease or disorder.

According to the present invention, substrates may include full length tyrosine phosphorylated proteins and polypeptides as well as fragments (*e.g.*, portions), derivatives or analogs thereof that can be phosphorylated at a tyrosine residue. Such fragments, derivatives and analogs include any PTP substrate polypeptide that retains at least the biological function of interacting with a PTP as provided herein, for example by forming a complex with a PTP. A fragment, derivative or analog of a PTP substrate polypeptide, including substrates that are fusion proteins, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue), and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the substrate polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (*e.g.*, polyethylene glycol) or a detectable moiety such as a reporter molecule, or (iv) one in which additional amino acids are fused to the substrate polypeptide, including amino acids that are employed for purification of the substrate polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art.

The subject invention also contemplates certain embodiments wherein the substrate trapping mutant PTP (that is combined with the sample) is a mutant PTP that is expressed by a cell, including embodiments wherein the cell has been transfected with one or more nucleic acid molecules encoding the mutant PTP. Thus, the method of identifying a tyrosine phosphorylated protein which is a substrate of a PTP may include in certain embodiments combining a sample comprising a tyrosine phosphorylated protein with a mutant PTP wherein the sample comprises a cell expressing either or both of the tyrosine phosphorylated protein and the mutant PTP.

Optionally, the cell may be transfected with nucleic acids encoding either or both of the tyrosine phosphorylated protein and the mutant PTP.

In another aspect, the invention provides methods of identifying an agent that alters the interaction between a PTP and a tyrosine phosphorylated protein that is a substrate of the PTP, through the use of screening assays that detect the ability of a candidate agent to alter (*i.e.*, increase or decrease) such interaction. The interaction between the PTP and its substrate may be determined enzymatically, for example by detecting catalytic substrate dephosphorylation. Alternatively, the interaction between the PTP (including a substrate trapping mutant PTP) and its substrate may be determined as a binding interaction, and in preferred embodiments such interaction is manifested as detection of a complex formed by PTP-substrate binding, according to criteria described herein. Agents identified according to these methods may be agonists (*e.g.*, agents that enhance or increase the activity of the wildtype PTP) or antagonists (*e.g.*, agents that inhibit or decrease the activity of the wildtype PTP) of PTP activity. Agents may be identified from among naturally occurring or non-naturally occurring compounds, including synthetic small molecules as described below.

In certain embodiments, wherein the screening assay is directed to PTP catalytic activity, the tyrosine phosphorylated protein that is a substrate of the PTP can be identified as described above, which method features the use of a novel substrate trapping mutant PTP as disclosed herein. Accordingly, a PTP and a tyrosine phosphorylated substrate are combined in the absence and in the presence of a candidate agent, where the substrate has first been identified as described above using a substrate trapping mutant PTP. The PTP and the substrate are combined under conditions permissive for the detectable dephosphorylation of the substrate to occur.

Any suitable method may be used to detect phosphoprotein dephosphorylation; such methods are well known in the art and include, without limitation, detection of substrate catalysis by one or more of, *e.g.*, radiometric, fluorimetric, densitometric, spectrophotometric, chromatographic, electrophoretic, colorimetric or biometric assays. The level of dephosphorylation of the substrate in the absence of the agent is compared to the level of dephosphorylation of the substrate in

the presence of the agent, such that a difference in the level of substrate dephosphorylation (*e.g.*, a statistically significant increase or decrease) indicates the agent alters the interaction between the protein tyrosine phosphatase and the substrate.

For instance, an enzymatic activity assay utilizing a wildtype PTP can be carried out in the absence and presence of a candidate agent. Enzymatic activity assays known in the art include, for example, PTP activity assays using a tyrosine phosphorylated ^{32}P-labeled substrate as described in Flint et al. (1993 *EMBO J.* *12*:1937-1946). A decrease in the PTP enzymatic activity in the presence of the candidate agent indicates that the agent inhibits the interaction between the PTP and its substrate. Conversely, an increase in PTP enzymatic activity in the presence of the agent indicates that the agent enhances the interaction between the PTP and its substrate.

In certain other embodiments, wherein the screening assay is directed to identifying an agent capable of altering a substrate trapping mutant PTP-substrate binding interaction, the substrate trapping mutant PTP (as described herein) and a tyrosine phosphorylated substrate are combined in the absence and in the presence of a candidate agent under conditions and for a time sufficient to permit formation of a complex between the tyrosine phosphorylated protein and the substrate trapping mutant PTP, thereby producing a combination. The formation of a complex comprising the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase in the combination is next determined (as also provided herein), wherein a difference between the level of complex formation (*e.g.*, a statistically significant difference) in the absence and in the presence of the agent indicates that the agent alters (*i.e.*, increases or decreases) the interaction between the protein tyrosine phosphatase and the substrate. Alternatively, a competitive binding assay can be carried out utilizing the substrate trapping mutant PTP in the absence and presence of a candidate agent. Competitive binding assays known in the art include, for example, U.S. Patent No. 5,352,660, which describes methods suitable for use according to these embodiments of the present invention. A decrease in the extent of PTP-substrate binding in the presence of the agent to be tested indicates that the agent inhibits the

interaction between the PTP and its substrate. Conversely, an increase in the extent of binding in the presence of the agent to be tested indicates that the agent enhances the interaction between the PTP and its substrate.

Candidate agents for use in a method of screening for an agent that alters the interaction between a PTP and its tyrosine phosphorylated protein substrate according to the present invention may be provided as "libraries" or collections of compounds, compositions or molecules. Candidate agents that may interact with one or more PTPs (including agents that interact with a substrate trapping mutant PTP as provided herein) may include members of phosphotyrosyl peptide libraries as described in Songyang et al. (1995 *Nature 373*:536-539; 1993 *Cell 72*:767-778) that bind to the PTP. Peptides identified from such peptide libraries can then be assessed to determine whether tyrosine phosphorylated proteins containing these peptides exist in nature. Alternatively, libraries of candidate molecules to be screened may typically include compounds known in the art as "small molecules" and having molecular weights less than 10^5 daltons, preferably less than 10^4 daltons and still more preferably less than 10^3 daltons. For example, members of a library of test compounds can be administered to a plurality of samples, each containing at least one substrate trapping mutant PTP and at least one tyrosine phosphorylated protein that is a substrate of the PTP as provided herein, and then assayed for their ability to enhance or inhibit mutant PTP binding to the substrate. Compounds so identified as capable of altering PTP-substrate interaction (*e.g.*, binding and/or substrate phosphotyrosine dephosphorylation) are valuable for therapeutic and/or diagnostic purposes, since they permit treatment and/or detection of diseases associated with PTP activity. Such compounds are also valuable in research directed to molecular signaling mechanisms that involve PTPs, and to refinements in the discovery and development of future compounds exhibiting greater specificity.

Candidate agents further may be provided as members of a combinatorial library, which preferably includes synthetic agents prepared according to a plurality of predetermined chemical reactions performed in a plurality of reaction vessels. For example, various starting compounds may be prepared employing one or more of solid-phase synthesis, recorded random mix methodologies and recorded reaction split

techniques that permit a given constituent to traceably undergo a plurality of permutations and/or combinations of reaction conditions. The resulting products comprise a library that can be screened followed by iterative selection and synthesis procedures, such as a synthetic combinatorial library of peptides (see *e.g.*, PCT/US91/08694, PCT/US91/04666, which are hereby incorporated by reference in their entireties) or other compositions that may include small molecules as provided herein (see *e.g.*, PCT/US94/08542, EP 0774464, U.S. 5,798,035, U.S. 5,789,172, U.S. 5,751,629, which are hereby incorporated by reference in their entireties). Those having ordinary skill in the art will appreciate that a diverse assortment of such libraries may be prepared according to established procedures, and tested using substrate trapping mutant PTPs according to the present disclosure.

The invention also pertains to a method of reducing the activity of a tyrosine phosphorylated protein, comprising administering to a subject a substrate trapping mutant PTP in which (i) the wildtype PTP catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (*e.g.*, an alanine residue), and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, whereby interaction of the substrate trapping mutant protein tyrosine phosphatase with the tyrosine phosphorylated protein reduces the activity of the tyrosine phosphorylated protein. In certain preferred embodiments, the tyrosine phosphorylated protein is VCP, $p130^{cas}$, the EGF receptor, p210 bcr:abl, MAP kinase, Shc or the insulin receptor. In certain other preferred embodiments, the protein tyrosine phosphatase is PTP1B, PTP-PEST, PTPγ, MKP-1, DEP-1, PTPμ, PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR or PTPH1.

Without wishing to be bound by theory, such a mutant PTP may reduce the activity of the corresponding wildtype PTP by forming a complex with the tyrosine phosphorylated protein substrate of the wildtype PTP, thereby rendering the substrate unavailable for catalytic dephosphorylation by the wildtype enzyme. The substrate trapping mutant PTP thus binds to the phosphoprotein substrate without

dephosphorylating it (or catalyzing dephosphorylation at a greatly reduced rate), thereby blocking the activity of the dephosphorylated protein substrate and reducing its downstream effects. As used herein, "reducing" includes both reduction and complete abolishment of one or more activities or functions of the phosphorylated protein substrate.

In one aspect of the method of reducing the activity of a tyrosine phosphorylated protein, a method is provided for reducing the transforming effects of at least one oncogene associated with phosphorylation of $p130^{cas}$, a substrate of PTP-PEST. The method generally comprises administering to a subject a substrate trapping mutant PTP-PEST in which the wildtype PTP catalytic domain invariant aspartate residue is replaced with an alanine residue, and in which at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. Whereas wildtype PTP-PEST binds and dephosphorylates the substrate $p130^{cas}$, thereby negatively regulating this substrate's downstream biological effects, the subject invention substrate trapping PTP-PEST mutants bind but cannot dephosphorylate $p130^{cas}$ (or do so at a greatly reduced rate). According to the non-limiting theory disclosed above, the substrate is thus sequestered in the complex with the substrate trapping PTP-PEST and cannot exert its downstream effects. In certain embodiments of this method, the oncogene may be one of v-crk, v-src or c-Ha-ras.

Similarly, the invention relates to a method of reducing the formation of signaling complexes associated with $p130^{cas}$, particularly those signaling complexes which induce mitogenic pathways, comprising administering to a mammal substrate trapping mutant PTP-PEST as provided above. The PTP binds to and/or dephosphorylates $p130^{cas}$, thereby negatively regulating the downstream effects of $p130^{cas}$ and reducing the formation of signaling complexes associated with $p130^{cas}$. As another example, in certain embodiments the invention relates to regulation of the cell cycle by the PTPH1 substrate pp97/VCP, wherein a substrate trapping mutant PTPH1 as provided herein (*i.e.*, a double mutant that is catalytically attenuated and in which a wildtype tyrosine has been replaced) can alter the interaction between PTPH1 and VCP.

As provided herein, the substrate trapping mutant PTPs of the present invention may be useful in virtually any situation where biological regulation involving PTP-regulated signal transduction is involved, for example, in place of, or in addition to, a corresponding wildtype PTP. The advantages of such utility of the subject invention lie in the ability of a substrate trapping mutant PTP to mimic the function of its corresponding wildtype enzyme, *e.g.*, to impair the biological signaling activity of a tyrosine phosphorylated substrate subsequent to dephosphorylation mediated by wildtype PTP, without inducing the harmful cytotoxic effects commonly observed when wildtype PTP is administered and/or overexpressed. Thus, the invention also pertains to a method of reducing the cytotoxic effects associated with administration or overexpression of wild type PTPs. For example, CS mutants of MKP-1 have been shown to have the same functional effect as wild type MKP-1 without induction of potentially harmful side effects. Thus, PTPs described herein, in which the wildtype PTP catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute (less than 1 min^{-1}) (*e.g.*, an alanine residue), and in which at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, can in many situations be substituted for a counterpart wildtype enzyme, where such a counterpart wildtype enzyme can specifically interact with the same substrate as the mutant PTP.

The substrate trapping mutant PTPs described herein may also be used therapeutically to alter (*i.e.*, increase or decrease) the activity of a tyrosine phosphorylated protein, such as by a gene therapy method in which a nucleic acid, for example, a recombinant expression construct as described above, encoding a substrate trapping mutant PTP (or a functional portion thereof) which retains the ability to bind to its tyrosine phosphorylated substrate, is introduced into a subject and is expressed. The mutant PTP replaces, either partially or totally, a corresponding host PTP enzyme that is normally produced in the subject, or may compete with the host PTP for binding to the substrate. For example, where a specific tyrosine phosphorylated protein substrate may be implicated in a particular disease or disorder, at least one PTP capable of

dephosphorylating the suspect substrate may be identified. A corresponding substrate trapping mutant PTP can be administered either directly or by gene therapy, using the compositions and methods described herein. Such a mutant PTP may sequester the tyrosine phosphorylated substrate, thereby inhibiting or reducing the substrate's role in the disease process. In a preferred embodiment, the substrate trapping mutant PTP of the present disclosure is administered in place of a corresponding wildtype enzyme, in order to reduce the cytotoxic effects associated with overexpression of the wild type enzyme. Procedures for gene therapy are known in the art (see, *e.g.*, U.S. Patent No. 5,399,346) and can be modified by known methods known in order to express the subject invention substrate trapping mutant PTPs.

The methods of the present invention are specifically exemplified herein with respect to the phosphatases PTPH1, PTP1B and PTP-PEST; however, it is understood that the invention is not limited to these specific PTPs but is applicable to all members of the PTP family. In order to identify potential substrates of PTPH1, PTP1B and PTP-PEST, mutant (*i.e.*, altered or substrate trapping) forms of PTPH1, PTP1B and PTP-PEST are generated as described herein that are catalytically attenuated but that retain the ability to bind substrates.

In certain embodiments, the invention relates in part to PTP1B(D181A), in which the aspartate residue at position 181 of wildtype PTP1B is replaced with alanine, and in which further a PTP tyrosine residue may optionally be replaced with a non-phosphorylatable residue. In certain other embodiments the invention relates to the phosphatase PTP-PEST(D199A) and in certain other embodiments to PTP-PEST(C231S), which in either case may further have a PTP tyrosine residue optionally replaced with a non-phosphorylatable residue. In particularly preferred embodiments the invention relates to PTPH1(Y676F/D811A).

As noted above, in certain embodiments the invention relates to a substrate trapping mutant PTP-PEST. PTP-PEST is an 86 kDa cytosolic PTP (Charest et al., 1995 *Biochem. J. 308*:425-432; den Hertog et al., 1992 *Biochem. Biophys. Res. Commun. 184*:1241-1249; Takekawa et al., 1992 *Biochem. Biophys. Res. Commun. 189*: 1223-1230; Yang et al., 1993 *J. Biol. Chem. 268*:6622-6628; Yang et al., 1992 *J. Biol.*

Chem. 268:17650) which is expressed ubiquitously in mammalian tissues (Yi et al., 1991 *Blood 78*:2222-2228), and which exhibits high specific activity when assayed *in vitro* using artificial tyrosine phosphorylated substrates (Garton and Tonks, 1994 *EMBO J. 13*:3763-3771). PTP-PEST is subject to regulation via phosphorylation of Ser39 *in vitro* and *in vivo*. This modification is catalyzed by both protein kinase C (PKC) and protein kinase A (PKA), and results in reduced PTP-PEST enzyme activity due to an increase in the Km for the dephosphorylation reaction catalyzed by this PTP (Garton and Tonks, 1994 *EMBO J. 13*:3763-3771). Additional intracellular regulatory mechanisms may include PTP-PEST-mediated dephosphorylation of one or more cytosolic substrates of tyrosine kinases.

As disclosed herein and described in the Examples, the substrate specificities of PTP1B and of PTPH1 may be characterized by methods that relate to PTP catalytic and/or binding interactions with substrate, *e.g.*, dephosphorylation and substrate trapping *in vitro* and *in vivo*. PTP1B (see, *e.g.*, Barford et al., 1994 *Science* 263:1397; Jia et al., 1995 *Science* 268:1754) and PTPH1 (see, *e.g.*, U.S. Patent Nos. 5,595,911 and 5,863,781) are well known in the art. The substrate trapping methods provided herein are generally applicable to any PTP by virtue of the invariant PTP catalytic domain aspartate residue and the frequency of tyrosine in PTP amino acid sequences, and should therefore prove useful in delineating the substrate preferences of other PTP family members. In particular, the use of mutant, catalytically impaired PTPs to trap, and thereby isolate, potential substrates permits the identification of physiologically important substrates for individual PTPs, leading to improved understanding of the roles of these enzymes in regulation of cellular processes. Furthermore, replacement of PTP tyrosine residues with amino acids that cannot be phosphorylated provides substrate trapping mutant PTPs that are not impaired in their ability to interact with tyrosine phosphorylated protein substrates.

The present invention also pertains to pharmaceutical compositions comprising a substrate trapping mutant PTP in which (i) the wildtype PTP catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat

to less than 1 per minute (less than 1 min^{-1}) (*e.g.*, an alanine residue); and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated (*e.g.*, not serine or threonine, nor any other naturally occurring or non-natually occurring amino that may be phosphorylated). The PTP of the present invention may therefore be formulated with a physiologically acceptable medium such as, for example, a pharmaceutically acceptable carrier or diluent, to prepare a pharmaceutical composition.

For administration to a patient, one or more polypeptides (including substrate trapping mutant PTPs), nucleic acid molecules (including recombinant expression constructs encoding substrate trapping mutant PTPs) and/or modulating agents (including agents that interact with a PTP and/or a substrate trapping mutant PTP) are generally formulated as a pharmaceutical composition. A pharmaceutical composition may be a sterile aqueous or non-aqueous solution, suspension or emulsion, which additionally comprises a physiologically acceptable carrier (*i.e.*, a non-toxic material that does not interfere with the activity of the active ingredient). Such compositions may be in the form of a solid, liquid or gas (aerosol). Alternatively, compositions of the present invention may be formulated as a lyophilizate or compounds may be encapsulated within liposomes using well known technology. Pharmaceutical compositions within the scope of the present invention may also contain other components, which may be biologically active or inactive. Such components include, but are not limited to, buffers (*e.g.*, neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.*, glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, stabilizers, dyes, flavoring agents, and suspending agents and/or preservatives.

Any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of the present invention. Carriers for therapeutic use are well known, and are described, for example, in *Remingtons Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro ed. 1985). In general, the type of carrier is selected based on the mode of administration. Pharmaceutical

compositions may be formulated for any appropriate manner of administration, including, for example, topical, oral, nasal, intraocular, intrathecal, rectal, vaginal, sublingual or parenteral administration, including subcutaneous, intravenous, intramuscular, intrasternal, intracavernous, intrameatal or intraurethral injection or infusion. For parenteral administration, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, kaolin, glycerin, starch dextrins, sodium alginate, carboxymethylcellulose, ethyl cellulose, glucose, sucrose and/or magnesium carbonate, may be employed.

A pharmaceutical composition (*e.g.*, for oral administration or delivery by injection) may be in the form of a liquid (*e.g.*, an elixir, syrup, solution, emulsion or suspension). A liquid pharmaceutical composition may include, for example, one or more of the following: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. A parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. The use of physiological saline is preferred, and an injectable pharmaceutical composition is preferably sterile.

The compositions described herein may be formulated for sustained release (*i.e.*, a formulation such as a capsule or sponge that effects a slow release of compound following administration). Such compositions may generally be prepared using well known technology and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain an agent dispersed in a carrier matrix and/or contained

within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active component release. The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

For pharmaceutical compositions comprising a nucleic acid molecule encoding a substrate trapping mutant PTP polypeptide (such that the polypeptide is generated *in situ*), the nucleic acid molecule may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid, and bacterial, viral and mammalian expression systems such as, for example, recombinant expression constructs as provided herein. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

Within a pharmaceutical composition, a substrate trapping mutant PTP polypeptide, a substrate trapping mutant PTP-encoding nucleic acid molecule or a modulating agent may be linked to any of a variety of compounds. For example, such a polypeptide, nucleic acid molecule or agent may be linked to a targeting moiety (*e.g.*, a monoclonal or polyclonal antibody, a protein or a liposome) that facilitates the delivery of the agent to the target site. As used herein, a "targeting moiety" may be any substance (such as a compound or cell) which, when linked to an agent, enhances the transport of the agent to a target cell or tissue, thereby increasing the local concentration of the agent. Targeting moieties include antibodies or fragments thereof, receptors, ligands and other molecules that bind to cells of, or in the vicinity of, the target tissue. An antibody targeting agent may be an intact (whole) molecule, a fragment thereof, or a functional equivalent thereof. Examples of antibody fragments are F(ab')2, -Fab', Fab and F[v] fragments, which may be produced by conventional methods or by genetic or

protein engineering. Linkage is generally covalent and may be achieved by, for example, direct condensation or other reactions, or by way of bi- or multi-functional linkers. Targeting moieties may be selected based on the cell(s) or tissue(s) at which the agent is expected to exert a therapeutic benefit.

Pharmaceutical compositions may be administered in a manner appropriate to the disease to be treated (or prevented). An appropriate dosage and a suitable duration and frequency of administration will be determined by such factors as the condition of the patient, the type and severity of the patient's disease, the particular form of the active ingredient and the method of administration. In general, an appropriate dosage and treatment regimen provides the agent(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit (*e.g.*, an improved clinical outcome, such as more frequent complete or partial remissions, or longer disease-free and/or overall survival). For prophylactic use, a dose should be sufficient to prevent, delay the onset of or diminish the severity of a disease associated with a defect in cell signaling, for example a defect leading to abnormal cell cycle regulation, proliferation, activation, differentiation, senescence, apoptosis, adhesion, metabolic activity, gene expression or the like.

Optimal dosages may generally be determined using experimental models and/or clinical trials. In general, the amount of polypeptide present in a dose, or produced *in situ* by DNA present in a dose, ranges from about 0.01 μg to about 100 μg per kg of host, typically from about 0.1 μg to about 10 μg. The use of the minimum dosage that is sufficient to provide effective therapy is usually preferred. Patients may generally be monitored for therapeutic or prophylactic effectiveness using assays suitable for the condition being treated or prevented, which will be familiar to those having ordinary skill in the art. Suitable dose sizes will vary with the size of the patient, but will typically range from about 1 mL to about 500 mL for a 10-60 kg subject.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated by reference in their entirety.

EXAMPLES

EXAMPLE 1

GENERATION, EXPRESSION AND PURIFICATION OF MUTANT PTP PROTEINS

Plasmid isolation, production of competent cells, transformation and related manipulations for the cloning, amplification, construction of recombinant plasmids, inserts and vectors, sequencing and the like, were carried out according to published procedures (Sambrook et al., *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; Ausubel et al., 1993 *Current Protocols in Molecular Biology*, Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., Boston, MA). Recombinant nucleic acid expression constructs encoding human PTP-PEST (Garton et al., 1994 *EMBO J.* 13:3763; Garton et al. 1996 *Mol. Cell. Biol.* 16:6408) and human PTP-1B (Brown-Shimer et al., 1990 Proc. Nat. Acad. Sci. USA 87:5148) were prepared as described.

Point mutations within the catalytic domains of PTPs were introduced using standard procedures, for example, the invariant aspartate (D) at amino acid position 199 in PTP-PEST being converted to alanine (A) by a substitution mutation (D199A). Thus, mutations giving rise to PTP-PEST(D199A), PTP-PEST(C231S), PTP1B(D181A) and PTP1B(C215S) were introduced by site-directed mutagenesis using the Muta-Gene™ *in vitro* mutagenesis kit (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. Regions containing the specified point mutation were then exchanged with the corresponding wild type sequences within appropriate expression vectors, and the replaced mutant regions were sequenced in their entirety to verify the absence of additional mutations.

Full length PTP-PEST proteins (wild type and mutant proteins, containing either Asp199 to Ala, or Cys231 to Ser mutations) and the wild type PTP-PEST catalytic domain (amino acids 1-305) were expressed in Sf9 cells using recombinant baculovirus (BaculoGold™, Pharmingen, San Diego, CA), and purified as described in Garton and Tonks (*EMBO J. 13*:3763-3771, 1994). Truncated forms of

wild type and mutant PTP-PEST proteins, comprising amino acid residues 1-305 of PTP-PEST were also expressed in *E. coli* as GST fusion proteins following subcloning of PTP-PEST DNA in-frame downstream of GST in pGEX vectors (Pharmacia Biotech Inc., Uppsala, Sweden). Twenty-five ml of *E. coli* transformed with the appropriate vector were grown to log phase (OD_{600} approximately 0.5). Fusion protein expression was then induced by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were grown for 2-4 hours at 30°C. Cells were harvested by centrifugation, incubated with 50 μg/ml lysozyme in 3 ml buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 0.1% Triton X-100 and 150 mM NaCl, then lysed by sonication (3 x 10s). Following removal of insoluble material by centrifugation (20 minutes at 300,000 x g), fusion proteins were isolated by incubation for 30 min at 4°C with 100 ml glutathione-Sepharose™ beads (Pharmacia Biotech Inc., Uppsala, Sweden), and the beads were then collected by centrifugation and washed three times with Buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10% glycerol, 1% Triton X-100 and 100 mM NaCl). This procedure yielded essentially homogeneous fusion protein at a concentration of 1 mg protein/ml glutathione-Sepharose beads. PTP1B proteins (wild type and mutant forms) comprising amino acids 1-321 were expressed in *E. coli* and purified to homogeneity as described in Barford et al. (*J. Mol. Biol. 239*:726-730 (1994)).

EXAMPLE 2

REGULATION OF PTP1B EXPRESSION LEVELS BY P210 BCR:ABL

Chronic myelogenous leukemia (CML) is a clonal disorder of the hematopoietic stem cell that is characterized by the Philadelphia chromosome (Ph), in which the c-Abl proto-oncogene on chromosome 9, encoding a protein tyrosine kinase (PTK), becomes linked to the bcr gene on chromosome 22. This results in the generation of a bcr:abl fusion protein, p210 bcr:abl in which the PTK activity is

enhanced relative to that of c-Abl. This example demonstrates that phosphorylation competent p210 bcr:abl protein specifically induces PTP1B expression.

When BaF3 cells (Jain et al., 1996 *Blood* 88:1542) expressing a temperature-sensitive mutant form of p210 bcr:abl were shifted to the permissive temperature for expression of p210 having PTK activity, PTP1B mRNA and protein expression levels were observed to increase within 12-24 hours, coincident with the appearance of the active form of the PTK (see, *e.g.*, WO98/04712; LaMontagne et al., 1998 *Mol. Cell. Biol.* 18:2965). The increase in expression of PTP1B was also observed in Philadelphia chromosome-positive (Ph+) B-lymphoid cells derived from a CML patient relative to Ph- cells from the same patient. Changes in PTP1B activity, which were commensurate with the change in enzyme protein levels, were also observed. These changes were specific for PTP1B and were not seen in the closely related homologue TC-PTP (which shares 65% amino acid sequence identity with PTP1B) or in other tested PTPs, including SHP-1, SHP-2 and PTP-PEST. The specificity of PTP1B induction by p210 bcr:abl PTK activity was confirmed using kinase-defective Rat1 cells (Pendergast et al., 1993 *Cell* 75:175). These cells express an inactive form of p210 bcr:abl, which contains an arginine instead of a lysine residue at amino acid position 1172 and which lacks PTK activity. Expression of this p210 mutant in Rat1 cells failed to result in altered PTP1B expression levels.

EXAMPLE 3

p210 BCR:ABL BINDING SUBSTRATE INTERACTIONS WITH A SUBSTRATE TRAPPING PTP MUTANT

This example describes exploitation of substrate interacting properties of a substrate trapping mutant PTP to identify a PTP substrate. Substrate trapping PTP polypeptides and fusion proteins were prepared as described in Example 1.

Substrate trapping mutant PTP polypeptides or fusion proteins were contacted with lysates derived from various cell lines. Briefly, as starting material for cell lysates, HeLa and COS cells were grown in Dulbecco's modified Eagle's medium

(DMEM), containing 5% fetal bovine serum (FBS); Rat1, Wi38, C2C12 and MvLu cells were grown in DMEM containing 10% FBS; 293 cells were grown in DMEM containing 10% calf serum; MCF10A cells were grown in 50% DMEM, 50% Ham's F-12 containing 5% horse serum, 20 ng/ml epidermal growth factor, 10 mg/ml insulin, 0.5 mg/ml hydrocortisone and 0.25 mg/ml fungizone; BaF3 cells were maintained as described (Jain et al., 1996 *Blood* 88:1542). All media also contained penicillin and streptomycin at 100 U/ml and 100 mg/ml, respectively, and all cells were grown at 37°C. Calcium phosphate-mediated transfection was used to introduce cDNA encoding wild type and mutant PTP-PEST proteins into COS cells. These were encoded by PTP-PEST cDNA (Garton et al., 1996 Mol. Cell. Biol. 16:6408) subcloned into the plasmid pMT2 (Sambrook et al., *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) from which expression was driven by an adenovirus major late promoter; 20 μg DNA was used for transfection of each 10 cm plate of cells. The level of expression of PTP-PEST constructs was similar in all cases.

Prior to cell lysis, 70-90% confluent cell cultures were treated for 30 minutes in medium containing 0.1 mM oxidized vanadate (pervanadate) (20 μl of a fresh solution containing 50 mM sodium metavanadate ($NaVO_3$) and 50 mM H_2O_2 added to 10 ml culture medium). Treatment of cells with H_2O_2 and vanadate leads to a synergistic increase in phosphotyrosine levels, presumably due to inhibition of intracellular PTPs by vanadate (Heffetz et al., 1990 *J. Biol. Chem.* 265:2896-2902). Pervanadate treatment resulted in the appearance of at least 50 prominent phosphotyrosine protein bands in all cell types, whereas untreated cells contained virtually undetectable levels of phosphotyrosine.

Cells were lysed in Buffer A (see Example 1) containing 5 mM iodoacetic acid. Following incubation at 4°C for 30 minutes, DTT was added to achieve a final concentration of 10 mM. Insoluble material was then removed by centrifugation for 20 minutes at 300,000 x g. The resultant lysates were stable with regard to their phosphotyrosine content during long term (several months) storage at -70°C and during prolonged (at least 20 hours) incubation at 4°C, in the absence of exogenous added PTPs.

Pervanadate-treated HeLa cell lysate was fractionated by anion exchange chromatography using a Mono Q FPLC column (Pharmacia). The sample (50 mg total protein at 3 mg/ml in buffer A) was diluted in three volumes of buffer B (20 mM tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 0.1% Triton X-100) prior to loading. Proteins were eluted at a flow rate of 1 ml/min with a linear gradient of 0-0.5 M NaCl in buffer B over 20 fractions (1 ml fraction volume), followed by a second gradient of 0.5-1.0 M NaCl in buffer B over 5 fractions. Phosphotyrosine-containing proteins were detected within fractions 7-21 according to anti-phosphotyrosine immunoblotting. The same procedures were followed for PTP1B, with the exception that the cells were not treated with pervanadate.

For dephosphorylation reactions, lysates of pervanadate-treated HeLa cells (1-2 mg protein/ml) containing tyrosine phosphorylated proteins were incubated on ice in the absence or presence of purified active PTPs at a concentration of 2 nM. Dephosphorylation was terminated by the removal of aliquots (30 μg protein) into SDS-PAGE sample buffer, and the extent of dephosphorylation was determined by immunoblotting using the phosphotyrosine-specific monoclonal antibody G104 generated as described below. Assays of PTP activity using tyrosine phosphorylated ^{32}P-labeled reduced and carboxyamidomethylated and maleylated lysozyme (RCM-lysozyme) as substrate were performed as described in Flint et al. (1993 *EMBO J.* *12*:1937-1946).

Antibodies and Immunoblotting: The PTP-PEST-specific monoclonal antibody AG25 was raised against baculovirus-expressed purified full-length PTP-PEST. The anti-phosphotyrosine monoclonal antibody G104 was generated using as antigen phosphotyrosine, alanine and glycine, in a 1:1:1 ratio, polymerized in the presence of keyhole limpet hemocyanin with 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide, a method originally described in Kamps and Sefton (*Oncogene* 2:305-315 (1988)). p130^{cas} monoclonal antibody was from Transduction Laboratories (Lexington, Ky). Monoclonal antibody FG6 against PTP1B was provided by Dr. David Hill (Calbiochem Oncogene Research Products, Cambridge, MA). Visualization of proteins by immunoblotting was achieved by enhanced

chemiluminescence (ECL) using HRP-conjugated secondary antibodies (Amersham Life Science Inc., Arlington Heights, Il) and the SuperSignal™ CL-HRP substrate system (Pierce, Rockford, Il).

Immunoprecipitation and Substrate Trapping: Immunoprecipitation of PTP-PEST from transfected COS cells was performed following covalent coupling of monoclonal antibody AG25 to protein A-Sepharose beads (Pharmacia Biotech Inc., Uppsala, Sweden) using the chemical cross-linking agent dimethyl pimelimidate (Schneider et al., *J. Biol. Chem.* 257:10766-10769 (1982)). Antibody was first bound to protein A-Sepharose at a concentration of 1 mg/ml bead volume, and unbound material was then removed by three washes with 0.2 M sodium borate, pH 9. Covalent coupling was achieved by incubation at room temperature for 30 minutes in the presence of 20 mM dimethyl pimelimidate in 0.2 M sodium borate, pH 9. The beads were then incubated for 1 hour with an excess of 0.2 M ethanolamine, pH 8, to block any unreacted cross-linker, and washed three times with PBS prior to storage at 4°C. Ten µl of AG25 beads were used to precipitate transfected PTP-PEST from lysates containing approximately 0.375 mg protein.

Substrate trapping was performed using various PTP affinity matrices. The full-length PTP-PEST matrix utilized covalent coupled AG25-protein A-Sepharose beads to which purified baculovirus-expressed PTP-PEST protein was bound. Aliquots (10 µl) of AG25 beads were incubated for 2 hours at 4°C in 100 µl buffer A in the presence of 5 µg of purified PTP-PEST (wild type or mutant forms); unbound PTP-PEST was then removed by washing three times with 1 ml buffer A. The resultant PTP-PEST-AG25-protein A-Sepharose beads contained approximately 2 mg of PTP-PEST per 10 ml aliquot. Substrate trapping was also carried out with glutathione-Sepharose beads bound to bacterially-expressed GST fusion proteins containing the catalytic domain of PTP-PEST.

PTP1B was also used in substrate trapping experiments. In this case, the monoclonal antibody FG6 was precoupled to protein A-Sepharose in the absence of cross-linker (2 µg antibody/10 µl beads), then purified PTP1B proteins were added in

excess and incubated at 4°C for 2 hours. Following removal of unbound PTP1B, 10 µl beads contained approximately 2 µg PTP1B.

Pervanadate-treated cell lysates, or column fractions, were used as a source of phosphotyrosine-containing proteins for substrate trapping experiments. In general, lysates containing 0.25-0.5 mg protein in 0.5 ml buffer A (including 5 mM iodoacetic acid, 10 mM DTT) were incubated at 4°C for 2 hours in the presence of 10 µl of affinity matrix containing approximately 2 µg of the appropriate PTP protein. Unbound proteins were then removed from the samples by washing three times with 1 ml buffer A, and bound material was collected by addition of 50 µl SDS-PAGE sample buffer followed by heating at 95°C for 5 minutes; proteins bound to the beads were then analyzed by SDS-PAGE followed by immunoblotting.

In transient cotransfection experiments in COS cells, PTP1B dephosphorylates p210 bcr:abl but not v-abl. When the PTP1B(D181A) mutant was expressed as a GST fusion protein, purified and incubated with lysates of Mo7-p210 cells (which overexpress p210 bcr:abl), a complex of the mutant PTP and p210 bcr:abl was isolated. In contrast, tyrosine phosphorylated c-abl, which was also present in the lysates, did not bind to the mutant PTP. The interaction between PTP1B(D181A) and p210 bcr:abl was blocked by vanadate, suggesting that the interaction involved the active site of the PTP.

Following transient coexpression in COS cells, PTP1B(D181A) formed a complex with p210 bcr:abl. The Y177F mutant form of p210 bcr:abl did not interact with PTP1B(D181A), suggesting that this tyrosine residue is a component of the binding site in the PTK. This tyrosine residue in p210 bcr:abl is phosphorylated *in vivo* and has been demonstrated to serve as a docking site for GRB2 (Pendergast et al., 1993 *Cell* 75:175). Direct interaction of the pTyr in p210 bcr:abl and the SH2 domain of GRB2 is essential for the transforming activity of the PTK. Interaction of PTP1B(D181A) with p210 bcr:abl interferes with the association of the PTK with GRB2. Taken together, these data suggest that p210 bcr:abl is a physiological substrate of PTP1B and that PTP1B may function as an antagonist of the oncoprotein PTK *in*

vivo. The Vmax, Km and Kcat of 37 kDa PTP1B mutants toward RCML are shown in Figure 2.

PTP1B and the EGF Receptor: Expression of PTP1B(D181A) in COS cells leads to enhanced phosphorylation of tyrosyl residues in a 180 kDa protein and in proteins of 120 and 70 kDa. When a GST-PTP1B(D181A) fusion protein is expressed in COS cells and precipitated on glutathione-Sepharose™, the 180 kDa, and smaller quantities of p120 and p70, were coprecipitated. The p180 protein was identified as the epidermal growth factor (EGF) receptor by immunoblotting. The identity of the p120 and p70 proteins is unclear; however, the latter is not src, p62 or paxillin.

Expression of PTP1B(D181A) in COS cells induces tyrosine phosphorylation of the EGF receptor in the absence of its ligand, EGF, indicating that the mutant PTP is exerting its effects in the intact cell and not post-lysis. The equivalent PTP-PEST(D199A) mutant, which has the corresponding aspartate at position 199 replaced with alanine, does not interact with the EGF receptor, indicating the specificity of this substrate interaction.

Autophosphorylation of the EGF receptor is required for the interaction with PTP1B(D181A). Mutants of the receptor that are either kinase-dead or in which the autophosphorylation sites have been deleted do not interact with PTP1B(D181A). In v-src-expressing cells, a plethora of tyrosine phosphorylated proteins were observed, but phosphorylation of the EGF receptor was not detected. Under these conditions, PTP1B D181A bound predominantly to a 70 kDa tyrosine phosphorylated protein. PTP1B thus appears capable of modulating EGF-induced signaling pathways.

EXAMPLE 4

PTP-PEST Preferentially Dephosphorylates a 130 kDa Phosphotyrosine Containing Protein

In order to investigate the substrate specificity of PTP-PEST *in vitro*, aliquots of pervanadate-treated HeLa cell lysates were incubated on ice, yielding 50-100 distinct phosphotyrosine-containing proteins as judged by immunoblotting of the cell

lysate using the monoclonal anti-phosphotyrosine antibody G104. Purified full-length PTP-PEST (expressed in Sf9 cells using recombinant baculovirus), PTP-PEST catalytic domain, or PTP1B catalytic domain (37 kDa form) was then added to the lysate, and aliquots were removed at various time points for analysis by SDS-PAGE followed by anti-phosphotyrosine immunoblotting.

Surprisingly, a prominent 130 kDa phosphotyrosine band (p130) was selectively dephosphorylated by PTP-PEST within 10 minutes, whereas the intensity of all the other bands was essentially unchanged even after 60 minutes of incubation with PTP-PEST. Long incubations with higher concentrations of PTP-PEST (greater than 100-fold) resulted in the complete removal of all phosphotyrosine bands from the lysate. However, under all conditions tested, p130 was found to be dephosphorylated more rapidly than all other bands present.

The selective dephosphorylation of p130 by PTP-PEST was also observed using a truncated form of the phosphatase (amino acid residues 1-305) which essentially contains only the catalytic domain of the enzyme. This result suggests that the striking substrate preference displayed by PTP-PEST in this analysis is an inherent property of the phosphatase catalytic domain, whereas the C-terminal 500 amino acid residues have little discernible effect on the substrate specificity of the enzyme.

The specificity of the interaction between PTP-PEST and p130 was examined using the catalytic domain of PTP1B (amino acid residues 1-321) in dephosphorylation reactions. When added at a similar molar concentration to that used for PTP-PEST, PTP1B was found to dephosphorylate fully and rapidly (within 15 minutes) most of the phosphotyrosine-containing proteins present in the pervanadate-treated HeLa lysate. In addition, the time course of dephosphorylation of p130 was not significantly more rapid than that of the other phosphotyrosine bands dephosphorylated by PTP1B. The range of PTP1B substrate specificity in vitro and in vivo thus can differ where availability of a given substrate may vary, and where an isolated PTP catalytic subunit is characterized.

EXAMPLE 5

IDENTIFICATION OF A 130 KDA SUBSTRATE OF PTP-PEST BY SUBSTRATE TRAPPING

This example describes the use of a substrate trapping mutant PTP in an affinity matrix, to identify a PTP substrate in a cell lysate. For preparation of the substrate trapping PTP affinity matrix, a mutant form of PTP-PEST (D199A) was generated by site-directed mutagenesis, and the mutant enzyme was purified following expression using recombinant baculovirus. When assayed using tyrosine phosphorylated RCM-Lysozyme as substrate, the purified mutant enzyme exhibited a specific activity which was approximately 10,000 fold lower than that of the wild type enzyme. This purified protein was bound to an affinity matrix comprised of an anti-PTP-PEST monoclonal antibody (AG25) covalently coupled to Protein A-Sepharose beads, then incubated with each of the Mono Q fractions prepared from HeLa cell lysates as described in Example 3.

Pervanadate-treated HeLa cell lysate was fractionated by anion exchange chromatography (Example 3) and aliquots of the fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-p130^{cas} antibodies. Aliquots of all samples analyzed were then incubated with an affinity matrix containing a substrate trapping PTP-PEST mutant, comprising full length PTP-PEST in which Asp199 is changed to alanine (D199A), bound to covalently coupled protein A-Sepharose/antibody (AG25) beads. After 45 minutes of incubation, proteins associating with the mutant PTP-PEST were collected by centrifugation, the beads were washed, and SDS-PAGE sample buffer was added. Associated proteins were then analyzed by immunoblotting using the monoclonal anti-phosphotyrosine antibody G104. Proteins associated with PTP-PEST were then analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-p130^{cas} antibodies.

Anti-phosphotyrosine immunoblotting of the column fractions showed that the p130 phosphotyrosine band eluted as a single peak in fractions 11-14 (approx. 0.3 M NaCl). In view of the abundance of tyrosine phosphorylated p130 in HeLa lysates, it appeared likely that p130 represents a previously identified phosphotyrosine-

containing 130 kDa protein. Several potential candidates were identified in the literature, including the focal adhesion kinase $p125^{FAK}$, ras-GAP, gp130 and $p130^{cas}$. Of these candidates, $p130^{cas}$ has been identified as a particularly prominent phosphotyrosine band in a wide variety of systems, including v-crk (Mayer and Hanafusa, *Proc. Natl. Acad. Sci. USA 87*:2638-2642 (1990); Mayer et al., *Nature 332*:272-275 (1988) and src (Kanner et al., *Proc. Natl. Acad. Sci. USA 87*:3328-3332 (1990); Reynolds et al., *Mol. Cell. Biol. 9*:3951-3958 (1989)) transformed fibroblasts, integrin-mediated cell adhesion (Nojima et al., *J. Biol. Chem. 270*:15398-15402 (1995); Petch et al., *J. Cell Science 108*:1371-1379 (1995); Vuori and Ruoslahti, *J. Biol. Chem. 270*:22259-22262 (1995)) and PDGF stimulated 3T3 cells (Rankin and Rozengurt, *J. Biol. Chem. 269*:704-710 (1994)).

Therefore, the possibility that the p130 phosphotyrosine band corresponds to $p130^{cas}$ was tested by immunoblotting the Mono Q fractions using an antibody to $p130^{cas}$. The 130 kDa band corresponding to $p130^{cas}$ eluted in the same fractions as the p130 tyrosine phosphorylated band, and displayed a similar apparent molecular weight, suggesting that they might represent the same protein. Furthermore, $p130^{cas}$ immunoprecipitated from these fractions was found to be phosphorylated on tyrosyl residues.

The mutant PTP-PEST protein was found to associate with a single phosphotyrosine-containing protein, the molecular weight (130 kDa) and Mono Q elution position (fractions 11-14) of which coincided with those of $p130^{cas}$. Immunoblotting of the PTP-PEST-associated proteins using the $p130^{cas}$ antibody demonstrated that the 130 kDa tyrosine phosphorylated protein trapped by the mutant PTP-PEST is indeed $p130^{cas}$. Therefore it appears that $p130^{cas}$ is a physiologically relevant substrate for PTP-PEST.

Structural Features of PTP-PEST in Specific Interaction with Tyrosine Phosphorylated $p130^{cas}$: The interaction between $P130^{cas}$ and PTP-PEST was investigated further in substrate trapping experiments using various purified mutant forms of PTP-PEST to precipitate proteins from pervanadate-treated HeLa lysates. Several affinity matrices were incubated with pervanadate-treated HeLa cell lysate, and

proteins associated with the beads were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine or anti-p130^{cas} antibodies.

The wild type full-length phosphatase was found to be incapable of stable association with tyrosine phosphorylated p130^{cas}, whereas both the PTP-PEST (D199A) mutant protein and a mutant lacking the active site cysteine residue (C231S) specifically precipitated p130^{cas} from the lysate. The inability of the wild type phosphatase to precipitate tyrosine phosphorylated p130^{cas} presumably reflects the transient nature of the normal interaction between PTP-PEST and tyrosine phosphorylated p130^{cas}, which is likely to be concluded as soon as p130^{cas} is dephosphorylated by PTP-PEST.

Since the C-terminal 500 amino acids of PTP-PEST contain several proline-rich regions which resemble src homology-3 (SH3) domain binding sequences. it appeared plausible that the specificity of the interaction between PTP-PEST and p130^{cas} might depend to some extent on association of these segments with the SH3 domain of p130^{cas}. The possible contribution of the C-terminal segment of PTP-PEST in the observed specific interaction of PTP-PEST with p130^{cas} was therefore addressed in further substrate trapping experiments using GST fusion proteins containing the catalytic domain of PTP-PEST alone, in both wild type and mutant (D199A) forms. The mutant catalytic domain of PTP-PEST fused to GST was found to precipitate the p130^{cas} phosphotyrosine band specifically, whereas both the wild type fusion protein and GST alone failed to precipitate p130^{cas}. The specific interaction between PTP-PEST and p130^{cas} observed in these experiments therefore appears to be an intrinsic property of the catalytic domain of PTP-PEST, emulating the observed preference of the active PTP-PEST catalytic domain for dephosphorylation of p130^{cas} *in vitro*.

Specificity of Interaction Between Mutant PTP-PEST and Tyrosine Phosphorylated p130^{cas}: In view of the relative abundance of tyrosine phosphorylated p130^{cas} in the pervanadate-treated HeLa cell lysate. the possibility that the observed selective binding of PTP-PEST inactive mutant proteins to p130^{cas} was substrate-directed (reflecting the abundance of this potential substrate relative to the other phosphotyrosine-containing proteins present in the lysate) rather than enzyme-directed

(reflecting a genuine substrate preference of PTP-PEST) was considered; this possibility was addressed in two ways. First, inactive mutant forms of the catalytic domain of PTP1B were used to trap potential substrates for this enzyme from the pervanadate-treated HeLa lysates. Again it was found that the wild type phosphatase was incapable of stable interaction with any phosphotyrosine-containing protein, whereas mutant variants of the PTP1B phosphatase domain (comprising Cys or Asp mutations analogous to those described above for PTP-PEST) associated with many tyrosine phosphorylated proteins. This was especially apparent for the aspartic acid mutant of PTP1B (D181A), which appeared to precipitate essentially all phosphotyrosine-containing proteins from the lysate with similar efficacy. These data emphasize the specific nature of the interaction between PTP-PEST and $p130^{cas}$, which appears to be a property peculiar to the PTP-PEST catalytic domain, rather than a feature shared by all PTP catalytic domains.

The specificity of the interaction between PTP-PEST and $p130^{cas}$ was addressed further following pervanadate-treatment of several different cell lines (Wi38, 293, COS, MCF10A, C2C12, MvLu), yielding a different array of tyrosine phosphorylated proteins in each case: the resultant lysates were analyzed by SDS-PAGE followed by anti-phosphotyrosine immunoblotting. Aliquots were incubated with PTP-PEST (D199A) affinity matrix or control matrix, and tyrosine phosphorylated proteins associating with PTP-PEST were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine or anti-$p130^{cas}$ antibodies as described above.

In each case, the D199A mutant PTP-PEST protein precipitated a single broad phosphotyrosine band with an apparent molecular weight between 120 and 150 kDa in different cell lines, whereas the affinity matrix alone failed to precipitate any phosphotyrosine-containing protein. Immunoblotting of the precipitates with a $p130^{cas}$ antibody revealed that the protein precipitated from all cell lysates corresponded to $p130^{cas}$; the observed molecular weight variation between different cell lines presumably reflects either species differences in the molecular weight of $p130^{cas}$ or expression of different alternatively spliced forms (Sakai et al., *EMBO J.* 13:3748-3756 (1994)).

The relative abundance of tyrosine phosphorylated p130^{cas} in the PTP-PEST precipitates appeared to correlate approximately with the abundance of p130^{cas} protein in the lysates (data not shown). Surprisingly, regardless of the abundance of tyrosine phosphorylated p130^{cas} in the lysates, p130^{cas} was invariably the only phosphotyrosine-containing protein in the precipitates, even in 293 cell lysates which contained very little p130^{cas} protein but which displayed a wide variety of other abundantly tyrosine phosphorylated proteins. Similarly, when lysates of pervanadate-treated 293 cells (containing tyrosine phosphorylated p130^{cas} in amounts which are undetectable by anti-phosphotyrosine immunoblotting of the lysate) were incubated with active PTP-PEST, no visible dephosphorylation of any phosphotyrosine band occurred (Garton and Tonks, unpublished data). These results indicate that the affinity of PTP-PEST for p130^{cas} is substantially greater than for any other substrate present, and further emphasizes the remarkable substrate selectivity of PTP-PEST for p130^{cas}.

Vanadate Inhibition of Tyrosine Phosphorylated p130^{cas} Association with Mutant PTP-PEST: A consistent observation was that, in contrast to the inactive mutant PTP-PEST, the wild type enzyme failed to associate in a stable complex with tyrosine phosphorylated p130^{cas}, suggesting that the observed association is active site-directed. In order to investigate this possibility, mutant PTP-PEST (D199A) was incubated with the PTP inhibitor vanadate (Denu et al., 1996 *Proc., Natl. Acad. Sci USA* *93*:2493-2498), at various concentrations prior to addition of pervanadate-treated HeLa cell lysate. The extent of association of p130^{cas} with PTP-PEST was then analyzed. PTP-PEST affinity matrix, comprising full length PTP-PEST (D199A) bound to covalently coupled protein A-Sepharose/antibody (AG25) beads, was incubated for 10 minutes on ice in the presence of varying concentrations of sodium orthovanadate. The samples were then incubated with aliquots of pervanadate-treated HeLa cell lysate: associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine or anti-p130^{cas} antibodies. The activity of wild type PTP-PEST was also determined under the same conditions, using tyrosine phosphorylated ^{32}P-labelled RCM-lysozyme as substrate.

The association was found to be potently disrupted by vanadate, with a concentration-dependence similar to that of vanadate inhibition of wild type PTP-PEST, and complete disruption being observed at 10 mM vanadate.

EXAMPLE 6

ASSOCIATION OF ENDOGENOUS p130^{CAS} WITH TRANSFECTED MUTANT PTP-PEST IN COS CELLS

The work described above strongly suggests that p130^{cas} represents a physiological substrate of PTP-PEST. In order to assess whether PTP-PEST interacts with p130^{cas} in intact cells, COS cells were transfected with plasmids encoding wild type or substrate trapping mutant forms (D199A or C231S) of PTP-PEST. The cells were treated with pervanadate 30 minutes prior to lysis. PTP-PEST proteins were immunoprecipitated, and associated tyrosine phosphorylated proteins were analyzed by anti-phosphotyrosine immunoblotting of the resultant precipitates. Lysates were also incubated with covalently coupled protein A-Sepharose/anti-PTP-PEST (AG25) beads and associated proteins were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody.

Under these conditions, the phosphotyrosine-containing band corresponding to p130^{cas} was again unique in its ability to associate with the C231S PTP-PEST protein, indicating that p130^{cas} can be specifically selected by PTP-PEST as a substrate in an intracellular context in the presence of a large number of alternative possible substrates. Neither the wild type nor the D199A form of PTP-PEST was capable of a stable interaction with tyrosine phosphorylated p130^{cas} in pervanadate-treated COS cells.

The binding of both wild type and D199A PTP-PEST to tyrosine phosphorylated p130^{cas} under these conditions is most likely prohibited by the presence of pervanadate bound to the active site cysteine residue of PTP-PEST (Denu et al., *Proc. Natl. Acad. Sci. USA* 93:2493-2498 (1996)), which effectively excludes the binding of phosphotyrosine residues of p130^{cas}. The ability of the C231S mutant PTP-

PEST to associate in a stable complex with p130^{cas} in the presence of pervanadate suggests that this mutant protein is largely unaffected by pervanadate, indicating that the normal mode of inhibition of PTPs by vanadate ions depends critically on direct interactions between vanadate and the thiolate anion of the PTP active-site cysteine residue. These observations therefore lend further support to the existence of an exclusive interaction between PTP-PEST and p130^{cas}, which appears exclusively to involve the PTP-PEST active site, and therefore reflects the physiological, highly restricted substrate preference of PTP-PEST for p130^{cas}.

EXAMPLE 7

PREPARATION OF SUBSTRATE TRAPPING PTP MUTANTS

Generation of mutant PTPs capable of interacting with substrates in a stable complex was essentially as described (Flint et al., 1997 *Proc. Nat. Acad. Sci.* USA *94*:1680; Garton et al., 1996 *Mol. Cell Biol. 16*:6408; Tiganis et al., 1997 *J. Biol. Chem. 272*:21548; *see also* PCT US97/13016). Plasmid isolation, production of competent cells, transformation and M13 manipulations were carried out according to published procedures (Sambrook et al., *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Purification of DNA fragments was achieved using a QIAEX™ kit purchased from QIAGEN, Inc. (Chatsworth, CA). Sequencing of the different constructs was performed using a Sequenase™ kit (Amersham-Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Restriction and modification enzymes were purchased from Roche Molecular Biochemicals (Indianapolis, IN) and New England Biolabs (Beverly, MA).

Briefly, human PTPH1 cDNA (U.S. Patent No. 5,595,911) ligated into plasmid pBlueScript (Stratagene, LaJolla, CA) was mutated by site-directed mutagenesis using the Muta-Gene™ kit (Bio-Rad, Inc., Hercules, CA) according to the supplier's instructions. The oligonucleotide used for *in vitro* mutagenesis of cysteine 842 to serine was:

CCT AGT TCA CTC CAG TGC TGG AAT AG SEQ ID NO:37

which spans nucleotides 2537-2562 of PTPH1. The oligonucleotide for mutagenesis of aspartate 811 to alanine was:

GCA TGG CCT GCC CAC GGT GTG C SEQ ID NO:38

which spans nucleotides 2445-2466 of PTPH1. The mutated replicative form DNA was transformed into *E. coli* strain DH10B (Stratagene, La Jolla, CA) and colonies were picked and dideoxy sequenced using a Sequenase™ kit (Amersham-Pharmacia, Piscataway, NJ) according to the manufacturer's instructions for verification of the mutation. The portions of the wildtype and mutated PTPH1 genes encoding the PTP catalytic domain (amino acid residues 634 to 913) were ligated in-frame into the expression vector pGEX (Amersham-Pharmacia, Piscataway, NJ) to generate three glutathione-S-transferase (GST) fusion protein encoding sequences: GST-PTPH1(wildtype), GST-PTPH1(D811A) and GST-PTPH1(C842S). GST-PTPH1 fusion proteins were expressed in *E. coli* and purified by affinity binding to glutathione immobilized on Sepharose™ beads (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol.

Alternatively, wildtype and mutant PTPH1 constructs as described above to be used for transfection of mammalian cells were tagged at the C-terminal encoding ends with nucleic acid sequences encoding the HA epitope. The HA tag corresponds to an antibody defined epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984 *Cell* 37:767):

SYPYDVPDYAS SEQ ID NO:39

After confirmation by DNA sequencing, these constructs were cloned into vector pCDNA3 (Invitrogen, Carlsbad, CA) and retroviral vector pBSTR1 (S. Reeves, Massachusetts General Hospital, Boston, MA).

PTPH1(D811A) mutant constructs were further modified by site directed mutagenesis as described above but using the oligonucleotide:

TTG GAC AAA AAC CGA TTT AAA GAT GTG CTG CCT TAT G SEQ ID NO:40

which spans nucleotides 2034-2070 of PTPH1 to generate a double mutant (Y676F/D811A) in which the conserved PTP catalytic site tyrosine residue at position 676 is replaced with phenylalanine.

EXAMPLE 8

INFLUENCE OF PTPH1 EXPRESSION ON CELL GROWTH IN TRANSFECTED CELLS

This example shows that overexpression of a transfected PTPH1 gene in cultured cells markedly impairs cell growth, while overexpression of a transfected mutant substrate-trapping PTPH1 gene does not.

Stable NIH3T3 cell lines expressing wildtype or substrate trapping mutant PTPH1 GST fusion proteins (see Example 7) under the control of a tetracycline-repressible promoter were constructed using a retroviral gene delivery system (Paulus et al., 1996 *J. Virol. 70*:62; Wang et al., 1998 *Genes Develop. 12*:1769). Briefly, confluent 10 cm diameter tissue culture plates of the viral packaging cell line LinX (G. Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were transfected by calcium-phosphate precipitation with 15 μg of either the wildtype or mutant D811A PTPH1 retroviral constructs. To maintain repression of PTPH1 gene expression, the following steps of establishing and maintaining the stable cell lines were performed in the presence of 2 μg/ml tetracycline (Clontech, Palo Alto, CA). Retroviruses were produced by culturing the transfected LinX cells at 30°C for 48 hours after which culture fluids containing virus were filtered using a 0.45 μm filter (Millipore, Bedford, MA) to remove packaging cells. The viral supernatants were supplemented with 4 μg/ml polybrene (Sigma, St. Louis, MO) and were used to infect NIH3T3 cells (Cold Spring Harbor Laboratory stock, originally obtained from American Type Culture

Collection, Rockville, MD) maintained in Dulbecco's modified Eagle's Medium (DMEM, GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO-BRL). Infection took place overnight at 30°C, after which the medium was replaced with fresh medium and cultures were incubated at 37°C. Two days later selective conditions were imposed by supplementing the medium with puromycin to a final concentration of 2 μg/ml. Individual colonies were isolated and maintained in the presence of both tetracycline and puromycin. To induce PTPH1 expression, cells were washed and re-seeded in new dishes in the presence of puromycin, but in the absence of tetracycline.

Cell growth was markedly inhibited (approximately seven-fold decrease in accumulated cell number) when wildtype PTPH1 catalytic domain expression was induced by removal of tetracycline from the culture media (Figures 3 and 4). Approximately 10% of the cells gradually detached from the culture dish during induction of wildtype PTPH1 expression, and these cells were non-viable as determined by their inability to exclude trypan blue. In contrast, expression of the catalytically impaired PTPH1-D811A mutant ("DA") had no effect on cell growth or viability. For each PTPH1 construct, similar results have been obtained in three separate cell lines generated from distinct isolated colonies, indicating that differences among clonal populations do not account for the phenotypic differences observed between cells transfected with wildtype and mutant (D811A) PTPH1. Using a DNA fragmentation assay (Wyllie, 1980 *Nature 284*:555; Arends et al., 1990 *Am. J. Pathol. 136*:593), it was determined that cells in which PTPH1 expression was induced did not undergo apoptosis.

Cell cycle analysis by flow cytofluorimetric measurement of DNA content using propidium iodide (Rabinovitch, 1994 *Meths. Cell Biol. 41*:263-296) was performed on populations of transfected cells in which wildtype or mutant (D811A) PTPH1 expression was induced. The distribution of cells amongst various phases of the cell cycle was not altered relative to control cells, indicating that PTPH1-induced growth arrest did not operate in a particular cell cycle phase.

Cells in culture were also synchronized to determine the effects of PTPH1 expression on re-entry into the cell cycle during recovery from G1/S arrest. Following a 24-hour period of induced PTPH1 (wildtype or mutant D811A) expression, cells were synchronized by cultivation for 18 h in the presence of 1 mM hydroxyurea (Calbiochem, San Diego, CA) ; this agent arrests cells at the G1/S boundary in the cell cycle (Kreck and DeCaprio, 1995 *Meths. Enzymol.* 254:114) . The hydroxyurea block was released by washing the cells with fresh medium three times. At various time points following removal of the cell cycle block, cells were lysed in NP40 buffer (1% NP40, 10 mM sodium phosphate-pH 7.0, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM benzamidine, 1 mM PMSF) for immunoblot analysis using a cyclin-specific antibody. Briefly, confluent cells in a 10 cm diameter tissue culture plate were lysed at 4°C for 10 min in 0.5 ml NP40 buffer, and the lysates were clarified by centrifugation at 10,000 x g for 10 min at 4°C. Aliquots of each lysate were normalized for protein concentration (BCA assay, Pierce Chemicals, Rockford, IL), diluted in sodium dodecylsulfate (SDS) sample buffer (Laemmli, 1970 Nature 227:680), resolved by SDS polyacrylamide gel electrophoresis using 8% acrylamide gels and blot transferred onto Immobilon-P PVDF membranes (Millipore, Bedford, MA). Polyclonal rabbit anti-cyclin D1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted according to the supplier's recommendations in immunoblot buffer (20 mM Tris-pH 7.5 containing 5% (w/v) nonfat dry milk, 150 mM NaCl and 0.05% Tween 20) were used to probe the blot for 1 hour at room temperature. The blot was washed three times in the same buffer and developed using enhanced chemiluminescence (ECL) reagents and horseradish peroxidase (HRP)-coupled secondary antibodies (both from Amersham-Pharmacia Biotech, Piscataway, New Jersey) according to the supplier's instructions, as previously described (Zhang et al., 1995 *J. Biol. Chem.* 270:20067).

As shown in Figure 5, when transfected cells were released from the hydroxyurea cell cycle block under conditions non-permissive for expression of the wildtype PTPH1 transgene, cyclin D expression gradually increased as cells reentered and progressed through the cell cycle. When, however, cells were released from the cell

cycle block under conditions permissive for PTPH1 expression, all detectable cyclin D expression was abolished, suggesting that PTPH1 retards cell growth by disrupting cell cycle progression. Expression of a mutant PTPH1(D811A) in cells transfected with the mutant transgene had no effect on the cell cycle.

EXAMPLE 9

IDENTIFICATION OF VCP AS A PTPH1 SUBSTRATE USING A PTPH1 SUBSTRATE TRAPPING MUTANT IN VITRO

This example describes identification of a PTPH1 substrate in cell lysates, using a substrate trapping PTPH1 mutant having the invariant PTP catalytic site aspartate residue replaced with alanine (D811A). Cell lysates were prepared as described above in Example 3, and then contacted with wildtype or mutant PTPH1 catalytic domains to determine PTP-substrate binding interactions.

Substrate trapping methodologies using mutant PTPs in which the invariant catlalytic domain asparate residue is replaced with alanine were as described (Flint et al., 1997 *Proc. Nat. Acad. Sci.* USA *94*:1680; Garton et al., 1996 *Mol. Cell Biol. 16*:6408; Tiganis et al., 1997 *J. Biol. Chem. 272*:21548; *see also* PCT US97/13016) except that the mutant PTP was PTPH1 (D811A) as described above in Example 8.

Pervanadate-treated cell lysates were incubated with GST-PTPH1 catalytic domain fusion proteins immobilized on Sepharose™ beads. Briefly, subconfluent mammalian cell cultures were treated with 50 µM pervanadate (diluted from a 1:1 mixture of 100 mM sodium vanadate and 100 mM H_2O_2 in DMEM) for 30 min, washed with PBS and lysed, as described in Example 8, in substrate-trapping buffer (1% Triton X-100, 50 mM HEPES-pH 7.5, 5 mM EDTA, 150 mM NaCl, 10 mM Na phosphate, 50 mM NaF, 5 mM iodoacetic acid, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM benzamidine and 1 mM PMSF. Lysates were made 10 mM DTT and clarified by centrifugation for 10 min at 10,000 x g. Purified GST-PTPH1 fusion proteins, or GST alone, bound to glutathione-Sepharose beads (Amersham-Pharmacia

Biotech, Piscataway, NJ) under conditions recommended by the supplier were extensively washed with phosphate buffered saline (PBS) containing 1% Triton X-100 (Sigma, St. Louis, MO), 2 mM diththiothreitol (DTT, Sigma), 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM benzamidine and 1 mM PMSF. Lysates were incubated with the bead-immobilized GST or GST-PTPH1 catalytic domain fusion proteins for 2 h at 4°C, and the beads were washed four times with substrate-trapping buffer. Material bound to the beads was resolved by SDS-PAGE and blotted onto Immobilon-P™ (Millipore, Bedford, MA) membranes, then probed with phosphotyrosine-specific monoclonal antibodies at concentrations recommended by the suppplier (G98, Tiganis et al., 1997 J. Biol. Chem. 272:21548; 4G10, Upstate Biotechnology, Lake Placid, NY; PY20, Transduction Laboratories, Lexington, KY) and developed using ECL reagents (Amersham-Pharmacia Biotech, Piscataway, NJ) as described above in Example 7.

A prominent, tyrosine-phosphorylated protein of 97 kDa (pp97) was specifically isolated by the PTPH1(D811A) mutant from 293 cell lysates, but not by either the wildtype PTPH1 or the PTPH1(C842S) mutant (Figure 6). Furthermore, pp97 was also consistently recovered by PTPH1(D811A) as the major tyrosine-phosphorylated protein from other mammalian cell lines tested, including A431, COS-7, HepG2, MDCK, REF-52, Saos-2 and Vero cells. The PTPH1 substrate trapping mutant specifically and preferentially bound to pp97, which was one of several hundred tyrosine-phosphorylated proteins present in the cell lysates; pp97 was not a major protein component in any of the cell lysates used as a starting material for substrate trapping. Variable amounts of other, minor tyrosine-phosphorylated proteins were also detected in the PTPH1-associated materials from the various cell lines.

Purification of pp97 on immobilized PTPH1(D811A) from lysates representing 10^8 293 cells was scaled up to obtain sufficient protein for partial sequencing by Edman degradation of K-endopeptidase-digested peptides (Russo et al., 1992 *J. Biol Chem.* 267:20317). Sequences of seven individual peptides were determined (Figure 7), all of which were found to match amino acid sequences present in a membrane-associated protein having ATPase activity and known as p97 or VCP (Egerton et al., 1992 *EMBO J.* 11:3533). Underlined sequences (Fig. 7) matched the

mouse VCP sequence retrieved from the NCBI database (http://www/ncbi.nlm.nih.gov/, accession number Z14044)(SEQ ID NO:42). The yeast ortholog of VCP, known as CDC48, is a well established cell cycle regulatory protein (Patel et al., 1998 *Trends Cell Biol.* 8:65). A synthetic peptide corresponding to the C-terminal 15 residues of murine VCP (Egerton et al., 1992) was prepared (Cold Spring Harbor Laboratory Core Peptide Facility, Cold Spring Harbor, NY) and conjugated using SPDP (N-succinimidyl 3-[2-pyridyldithio]proprionate, Pierce Chemicals, Rockford, IL) according to the manufacturer's recommendations to keyhole limpet hemocyanin (KLH, Pierce Chemicals) for use as an immunogen according to standard procedures (Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988; Weir, D.M., *Handbook of Experimental Immunology*, 1986, Blackwell Scientific, Boston) to generate polyclonal rabbit antiserum CS531. The VCP peptide immunogen had the sequence:

GGSVYTEDNDDDLYG SEQ ID NO:41

EXAMPLE 10

IDENTIFICATION OF VCP AS A PTPH1 SUBSTRATE USING A SUBSTRATE TRAPPING PTPH1 DOUBLE MUTANT HAVING SUBSTITUTED ACTIVE SITE TYROSINE

This example describes identification of an interaction between a PTP and its substrate in intact cells, using a substrate trapping PTP double mutant. More specifically, this example employs the PTPH1 double mutant having the invariant PTP catalytic site aspartate residue replaced with alanine (D811A) and also having the conserved PTP catalytic site tyrosine residue at position 676 is replaced with phenylalanine.

Cultured 293 cells were transfected using the HA-tagged PTPH1 constructs described in Example 8, and the expressed HA epitope tagged proteins were recovered by immunoprecipitation with HA-specific monoclonal antibody 12CA5 bound to immobilized staphylococcal protein A as described (Zhang et al., 1997 *J. Biol.*

Chem. 272:27281). Immunoprecipitates were prepared according to standard procedures from lysates produced as described above in Example 3. Immunoprecipitates were analyzed for phosphotyrosine-containing proteins by western immunoblot methods as described above. Surprisingly, the PTPH1(D811A) mutant expressed in 293 cells contained significant and readily detectable levels of phosphotyrosine (Figure 8A), which contrasted with the absence of detectable phosphotyrosine in the GST-PTPH1(D811A) fusion protein expressed in *E. coli* (Figure 6). From these results, the location of phosphorylated tyrosine in the PTPH1 primary structure could not be determined. Additionally, the PTPH1(D811A) mutant expressed in 293 cells did not co-precipitate detectable pp97/VCP (Figure 8). PTPH1(D811A) thus failed to efficiently trap detectable pp97/VCP *in vivo* in a manner commensurate with the *in vitro* pp97/VCP trapping exhibited by PTPH1(D811A) in vitro (Example 9).

Analysis of the PTPH1 catalytic domain amino acid sequence revealed the presence of a conserved tyrosine residue at position 676 in the PTP active site (Barford et al., 1995 *Nat. Struct. Biol.* 2:1043). An HA-tagged PTPH1 double mutant was constructed as described in Example 8, in which the tyrosine at position 676 of PTPH1(D811A) was replaced with phenylalanine to provide PTPH1(Y676F/D811A). Cell lysates from 293 cells transfected with a construct encoding the PTPH1 (Y676/D811A) double mutant were lysed, immunoprecipitated with monoclonal anti-HA antibody and analyzed by western immunoblot methodologies as described above for the presence of phosphotyrosine. Immunoprecipitated materials were also analyzed for the presence of pp97/VCP using antiserum CS531 (Example 9), and for the presence of the HA epitope using monclonal antibody 12CA5 (Zhang et al., 1997 *J. Biol. Chem.* 272:27281).

Unlike the 293 cells transfected with the single mutant PTPH1(D811A), 293 cells transfected with the double mutant PTPH1(Y676F/D811A) had gained the ability to specifically trap pp97/VCP, as demonstrated by immunoblot analysis of the immunoprecipate using antiserum CS531 (Figure 8). When analyzed for phosphotyrosine content, the double mutant PTPH1(Y676F/D811A)

immunoprecipitated from transfected 293 cells exhibited dramatically reduced phosphotyrosine, relative to the single mutant PTPH1(D811A) (Figure 8B).

EXAMPLE 11

IDENTIFICATION OF TYROSINE PHOSPHORYLATION SITES ON A PTPH1 SUBSTRATE IN VIVO USING A SUBSTRATE TRAPPING PTPH1 DOUBLE MUTANT

In this example, a substrate trapping PTPH1 double mutant is used to functionally characterize tyrosine phosphorylation sites on pp97/VCP. The tyrosines (Y796 and Y805) at the C-terminus of VCP are major phosphorylation sites that may be responsible for VCP roles in cell cycle regulation via heretofore uncharacterized molecular pathways (Egerton et al., 1994 *J. Biol. Chem. 269*:11435; Madeo et al., 1998 *Mol. Biol. Cell 9*:131).

Human 293 cells were co-transfected with (i) one of the HA-tagged PTPH1 constructs (wildtype, D811A or Y676F/D811A) as described in Examples 8-10, and (ii) either a wildtype VCP construct (VCPmyc) or a double mutant (Y796F/Y805F) VCP construct (VCPmyc-FF, L. Samelson, National Institutes of Health, Bethesda, Maryland) in which the two C-terminal tyrosine phosphorylation sites are replaced with phenylalanines. The VCP wildtype and mutant constructs were tagged with the Myc epitope recognized by monoclonal antibody 9E10 (American Type Culture Collection, Rockville, Maryland). Co-transfected cells were lysed as described in Example 3 and immunoprecipitated with antibody 12CA5 (anti-HA) as described (Zhang et al., 1997 *J. Biol. Chem. 272*:27281).

Electrophoretically resolved and blotted components were then probed with anti-myc antibody 9E10 to identify VCP proteins that co-precipitated with (*i.e.*, were "trapped" by) the PTPH1 protein, or with anti-HA to confirm the presence of PTPH1 proteins in immunoprecipitated material. Wildtype and mutant PTPH1 proteins were expressed at comparable levels, as were the two forms of VCP. The PTPH1(Y676F/D811A) double mutant trapped wildtype VCP efficiently, but did not trap the double mutant VCP, which lacks two C-terminal tyrosine phosphorylation sites

(Figure 9). Also, neither wildtype PTPH1, nor the single mutant PTPH1(D811A), effectively trapped VCP (Figure 9).

EXAMPLE 12

SELECTIVE DEPHOSPHORYLATION OF VCP BY PTPH1

In this example, the effect of PTPH1 on the phosphorylation state of VCP was examined. Stable NIH3T3 cells, transfected with and expressing full length wildtype PTPH1 under control of a tetracycline-repressible promoter in the presence or absence of tetracycline, were pretreated with 1 mM vanadate for 1 hour prior to lysis, and VCP was immunoprecipitated using rabbit CS531 antiserum. Lysates and immunoprecipitates were prepared according to standard procedures as described above in Example 3, except cells were lysed in RIPA buffer (NP40 buffer supplemented with 1% sodium deoxycholate and 0.1% SDS) instead of NP40 buffer. Under conditions permissive for PTPH1 expression (+), a three- to five-fold decrease in the phosphotyrosine level of VCP was observed, relative to that seen when PTPH1 expression was repressed (-) (Figure 10A).

Lysates from the NIH3T3 transfectants were also immunoprecipitated with anti-phosphotyrosine antibody PT66 (Sigma, St. Louis, MO) to obtain a representative sample of tyrosine-phosphorylated proteins from cells cultured in the presence (+) or absence (-) of PTPH1 expression (Figure 10B). Immunoblot analysis of these immunoprecipitates with antibodies specific for VCP revealed dramatically reduced levels of VCP among tyrosine-phosphorylated proteins immunoprecipitated from cells in which PTPH1 expression was induced (+) relative to uninduced controls (-) (Figure 10B). The apparently selective dephosphorylation of VCP by PTPH1 in these cells was also shown by assessing the effect of PTPH1 induction on the degree of tyrosine phosphorylation of a distinct tyrosine-phosphorylated protein, the kinase FAK. Induction of PTPH1 expression did not cause a corresponding decrease in the level of FAK that was immunoprecipitated by anti-phosphotyrosine antibodies (Figure 10B).

The effects of induced PTPH1 expression on total tyrosine-phosphorylated protein pools were also compared in PTPH1-transfected NIH3T3 cells grown under normal conditions ("untreated"), under serum starvation by cultivation in DMEM containing 0.5% FBS for 16 hours ("starved") or following insulin stimulation of starved cells by 10 μg/ml insulin (Roche Molecular Biochemicals, Indianapolis, IN) for 10 minutes. Aliquots of total cell lysates were electrophoretically resolved, blot transferred to Immobilon-P™ and probed with a mixture of two HRP-conjugated anti-phosphotyrosine antibodies, PY20 (Transduction Laboratories, Lexington, KY) and 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) diluted according to the suppliers' recommendations, followed by ECL detection (Amersham, Cleveland, OH). The induction of PTPH1 overexpression failed to alter the global pattern of protein tyrosine phosphorylation in randomly growing ("untreated"), starved or insulin-stimulated cells (Figure 11).

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. Also, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the present invention is not limited except as by the appended claims.

CLAIMS

What is claimed is:

1. A substrate trapping mutant protein tyrosine phosphatase in which

a) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and

b) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

2. The substrate trapping mutant of claim 1 in which at least one wildtype tyrosine residue is replaced with an amino acid selected from the group consisting of alanine, cysteine, aspartic acid, glutamine, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, arginine, valine and tryptophan.

3. The substrate trapping mutant of claim 1 wherein at least one tyrosine residue that is replaced is located in a protein tyrosine phosphatase catalytic domain.

4. The substrate trapping mutant of claim 1 wherein at least one tyrosine residue that is replaced is located in a protein tyrosine phosphatase active site.

5. The substrate trapping mutant protein tyrosine phosphatase of claim 1 wherein at least one tyrosine residue is replaced with phenylalanine.

6. The substrate trapping mutant protein tyrosine phosphatase of claim 1 wherein at least one tyrosine residue that is replaced is a protein tyrosine phosphatase conserved residue.

7. The substrate trapping mutant of claim 6 wherein the conserved residue corresponds to tyrosine at amino acid position 676 in human PTPH1.

8. The substrate trapping mutant of claim 1 wherein at least one tyrosine residue is replaced with an amino acid that stabilizes a complex comprising the protein tyrosine phosphatase and at least one substrate molecule.

9. The substrate trapping mutant of claim 1 comprising a mutated PTPH1.

10. The substrate trapping mutant of claim 1 comprising a mutated protein tyrosine phosphatase selected from the group consisting of PTP1B, PTP-PEST, PTPγ, MKP-1, DEP-1, PTPμ, PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR and PTPH1.

11. The substrate trapping mutant of claim 1 comprising a mutated PTP-PEST phosphatase in which the amino acid at position 231 is replaced with a serine residue.

12. A method of identifying a tyrosine phosphorylated protein which is a substrate of a protein tyrosine phosphatase, comprising the steps of:

a) combining a sample comprising at least one tyrosine phosphorylated protein with at least one substrate trapping mutant protein tyrosine phosphatase, in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, under conditions and for a time sufficient to permit formation of a complex between the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase; and

b) determining the presence or absence of a complex comprising the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine

phosphatase, wherein the presence of the complex indicates that the tyrosine phosphorylated protein is a substrate of the protein tyrosine phosphatase with which it forms a complex.

13. A method according to claim 12, wherein the substrate trapping mutant comprises a mutated protein tyrosine phosphatase that is selected from the group consisting of PTP1B, PTP-PEST, PTP-γ, MKP-1, DEP-1, PTP-μ, PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR and PTPH1.

14. The method of claim 12 wherein the sample comprises a cell that expresses the tyrosine phosphorylated protein.

15. The method of claim 14 wherein the cell has been transfected with at least one nucleic acid molecule encoding the substrate.

16. The method of claim 12 wherein at least one substrate trapping mutant protein tyrosine phosphatase is expressed by a cell.

17. The method of claim 16 wherein the cell has been transfected with at least one nucleic acid molecule encoding the substrate trapping mutant protein tyrosine phosphatase.

18. The method of claim 12 wherein the sample comprises a cell that expresses (i) the tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase, and (ii) the substrate trapping mutant protein tyrosine phosphatase.

19. The method of claim 18 wherein the cell has been transfected with (i) at least one nucleic acid encoding the substrate, and (ii) at least one nucleic acid encoding the substrate trapping mutant protein tyrosine phosphatase.

20. The method of claim 12 wherein the sample comprises a cell lysate containing at least one tyrosine phosphorylated protein.

21. The method of claim 20 wherein the cell lysate is derived from a cell transfected with at least one nucleic acid encoding the tyrosine phosphorylated protein.

22. The method of claim 20 wherein the cell lysate is derived from a cell transfected with at least one nucleic acid encoding a protein tyrosine kinase.

23. The method of claim 12 wherein at least one substrate trapping mutant protein tyrosine phosphatase is present within a cell lysate.

24. The method of claim 23 wherein the cell lysate is derived from a cell transfected with at least one nucleic acid encoding the substrate trapping mutant protein tyrosine phosphatase.

25. A method according to claim 12 wherein the tyrosine phosphorylated protein is selected from the group consisting of VCP, $p130^{cas}$, the EGF receptor, p210 bcr:abl, MAP kinase, Shc and the insulin receptor.

26. A method of identifying an agent which alters the interaction between a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase, comprising:

(a) contacting in the absence and in the presence of a candidate agent, a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase under conditions and for a time sufficient for detectable dephosphorylation of the substrate to occur, wherein the tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase is identified by (1) combining a sample comprising at least one tyrosine phosphorylated protein with at least one substrate trapping mutant protein tyrosine phosphatase, in which (i) the wildtype protein tyrosine

phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, under conditions and for a time sufficient to permit formation of a complex between the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase; and (2) determining the presence or absence of a complex comprising the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase, wherein the presence of the complex indicates that the tyrosine phosphorylated protein is a substrate of the protein tyrosine phosphatase with which it forms a complex; and

(b) comparing the level of dephosphorylation of the substrate in the absence of the agent to the level of dephosphorylation of the substrate in the presence of the agent, wherein a difference in the level of substrate dephosphorylation indicates the agent alters the interaction between the protein tyrosine phosphatase and the substrate.

27. A method of identifying an agent which alters the interaction between a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase, comprising:

(a) contacting in the absence and in the presence of a candidate agent, a protein tyrosine phosphatase and a tyrosine phosphorylated protein which is a substrate of the protein tyrosine phosphatase under conditions and for a time sufficient to permit formation of a complex between the tyrosine phosphorylated protein and the substrate trapping mutant protein tyrosine phosphatase, wherein the substrate trapping mutant protein tyrosine phosphatase comprises a mutated protein tyrosine phosphatase in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated; and

(b) comparing the level of complex formation in the absence of the agent to the level of complex formation in the presence of the agent, wherein a difference in the

level of complex formation indicates the agent alters the interaction between the protein tyrosine phosphatase and the substrate.

28. A method of reducing the activity of a tyrosine phosphorylated protein comprising administering to a subject a substrate trapping mutant of a protein tyrosine phosphatase in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated, whereby interaction of the substrate trapping mutant protein tyrosine phosphatase with the tyrosine phosphorylated protein reduces the activity of the tyrosine phosphorylated protein.

29. A method according to claim 28, wherein the tyrosine phosphorylated protein is selected from the group consisting of VCP, $p130^{cas}$, the EGF receptor, p210 bcr:abl, MAP kinase, Shc and the insulin receptor.

30. A method according to claim 28, wherein the protein tyrosine phosphatase is selected from the group consisting of PTP1B, PTP-PEST, PTPγ, MKP-1, DEP-1, PTPμ, PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR and PTPH1.

31. A method of reducing a transforming effect of at least one oncogene associated with $p130^{cas}$ phosphorylation comprising:

administering to a mammal capable of expressing $p130^{cas}$ a substrate trapping mutant of PTP-PEST in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated:

whereby the substrate trapping mutant interacts with $p130^{cas}$ to reduce the transforming effect of at least one oncogene associated with $p130^{cas}$ phosphorylation.

32. A method according to claim 31 wherein the oncogene is selected from the group consisting of v-crk, v-src and c-Ha-ras.

33. A method of reducing formation of signaling complexes associated with $p130^{cas}$, comprising administering to a mammal capable of expressing $p130^{cas}$ a substrate trapping mutant of PTP-PEST in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated;

whereby the substrate trapping mutant interacts with $p130^{cas}$ to reduce the formation of signaling complexes associated with $p130^{cas}$.

34. A method of reducing cytotoxic effects associated with protein tyrosine phosphatase administration or overexpression, comprising administering to a mammal a substrate trapping mutant of a protein tyrosine phosphatase in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

35. An isolated nucleic acid molecule encoding a substrate trapping mutant protein tyrosine phosphatase in which

a) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and

b) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

36. An antisense oligonucleotide comprising at least 15 consecutive nucleotides complementary to the nucleic acid molecule of claim 35.

37. A fusion protein comprising a polypeptide sequence fused to a substrate trapping mutant protein tyrosine phosphatase in which

a) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and

b) at least one wildtype protein tyrosine phosphatase tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

38. The fusion protein of claim 37 wherein the polypeptide is an enzyme or a variant or fragment thereof.

39. The fusion protein of claim 37 wherein the polypeptide sequence fused to a substrate trapping mutant protein tyrosine phosphatase is cleavable by a protease.

40. The fusion protein of claim 37 wherein the polypeptide sequence is an affinity tag polypeptide having affinity for a ligand.

41. A recombinant expression construct comprising at least one promoter operably linked to a nucleic acid encoding a substrate trapping mutant protein tyrosine phosphatase in which

a) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute: and

b) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

42. The expression construct of claim 41 wherein the promoter is a regulated promoter.

43. An expression construct according to claim 41 wherein the substrate trapping mutant protein tyrosine phosphatase is expressed as a fusion protein with a polypeptide product of a second nucleic acid sequence.

44. The expression construct of claim 43 wherein the polypeptide product of said second nucleic acid sequence is an enzyme.

45. A recombinant expression construct according to claim 41 wherein the expression construct is a recombinant viral expression construct.

46. A host cell comprising a recombinant expression construct according to any one of claims 41–45.

47. A host cell according to claim 46 wherein the host cell is a prokaryotic cell.

48. A host cell according to claim 46 wherein the host cell is a eukaryotic cell.

49. A method of producing a recombinant substrate trapping mutant protein tyrosine phosphatase. comprising:

culturing a host cell comprising a recombinant expression construct comprising at least one promoter operably linked to a nucleic acid sequence encoding a substrate trapping mutant protein tyrosine phosphatase in which

a) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and

b) at least one wildtype protein tyrosine phosphatase tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

50. The method of claim 48 wherein the promoter is a regulated promoter.

51. A method of producing a recombinant substrate trapping mutant protein tyrosine phosphatase, comprising:

culturing a host cell infected with the recombinant viral expression construct of claim 45.

52. A pharmaceutical composition comprising:

a substrate trapping mutant protein tyrosine phosphatase in which

a) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute; and

b) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

in combination with a pharmaceutically acceptable carrier or diluent.

53. A pharmaceutical composition comprising an agent that interacts with a substrate trapping mutant protein tyrosine phosphatase in which

a) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute: and

b) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated.

in combination with a pharmaceutically acceptable carrier or diluent.

54. A kit for identifying a tyrosine phosphorylated protein substrate of a protein tyrosine phosphatase comprising:

a) at least one substrate trapping mutant protein tyrosine phosphatase in which (i) the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the Km of the enzyme but which results in a reduction in Kcat to less than 1 per minute, and (ii) at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated; and

b) ancillary reagents suitable for use in detecting the presence or absence of a complex between the protein tyrosine phosphatase and a tyrosine phosphorylated protein.

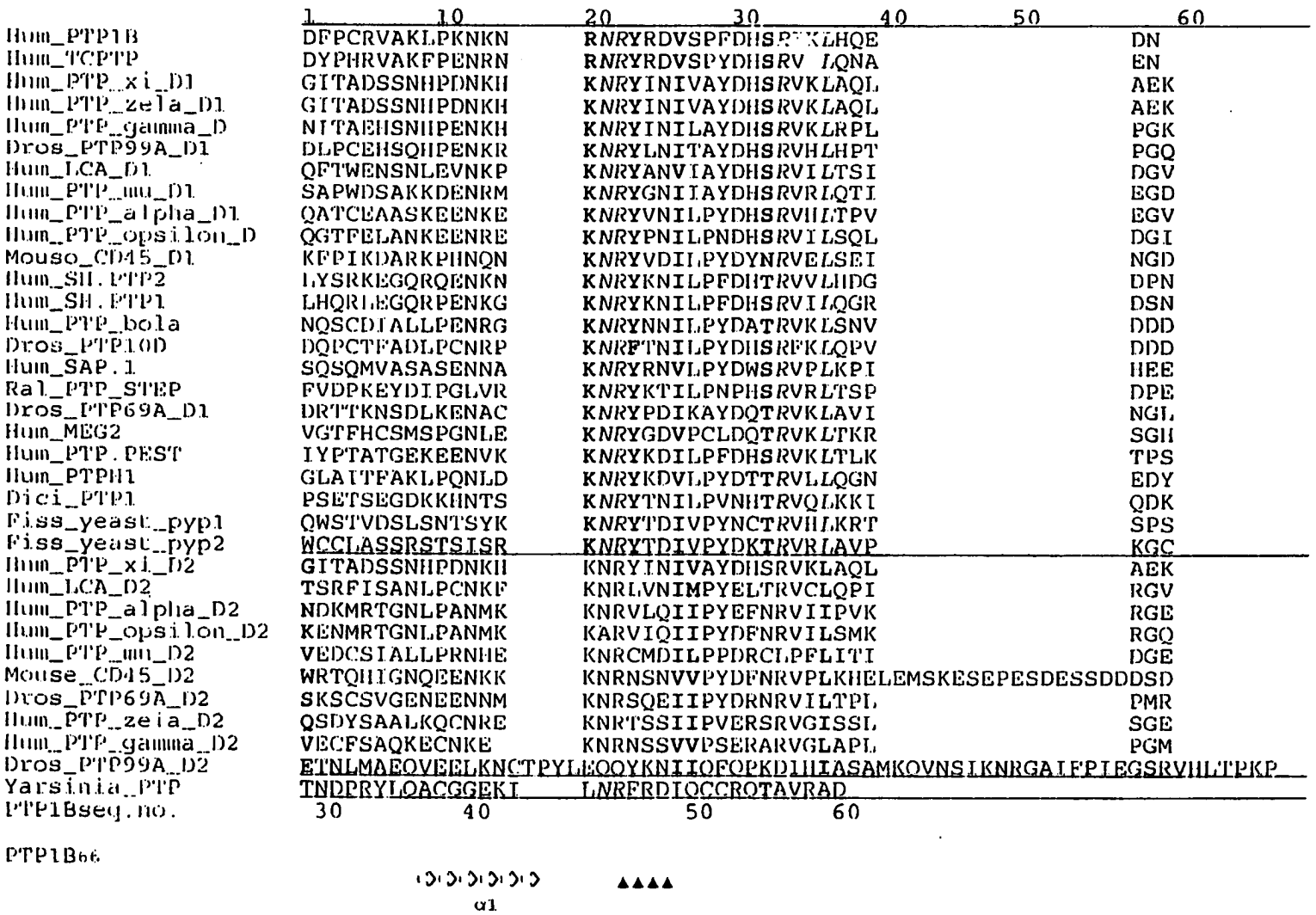
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FIG. 1A

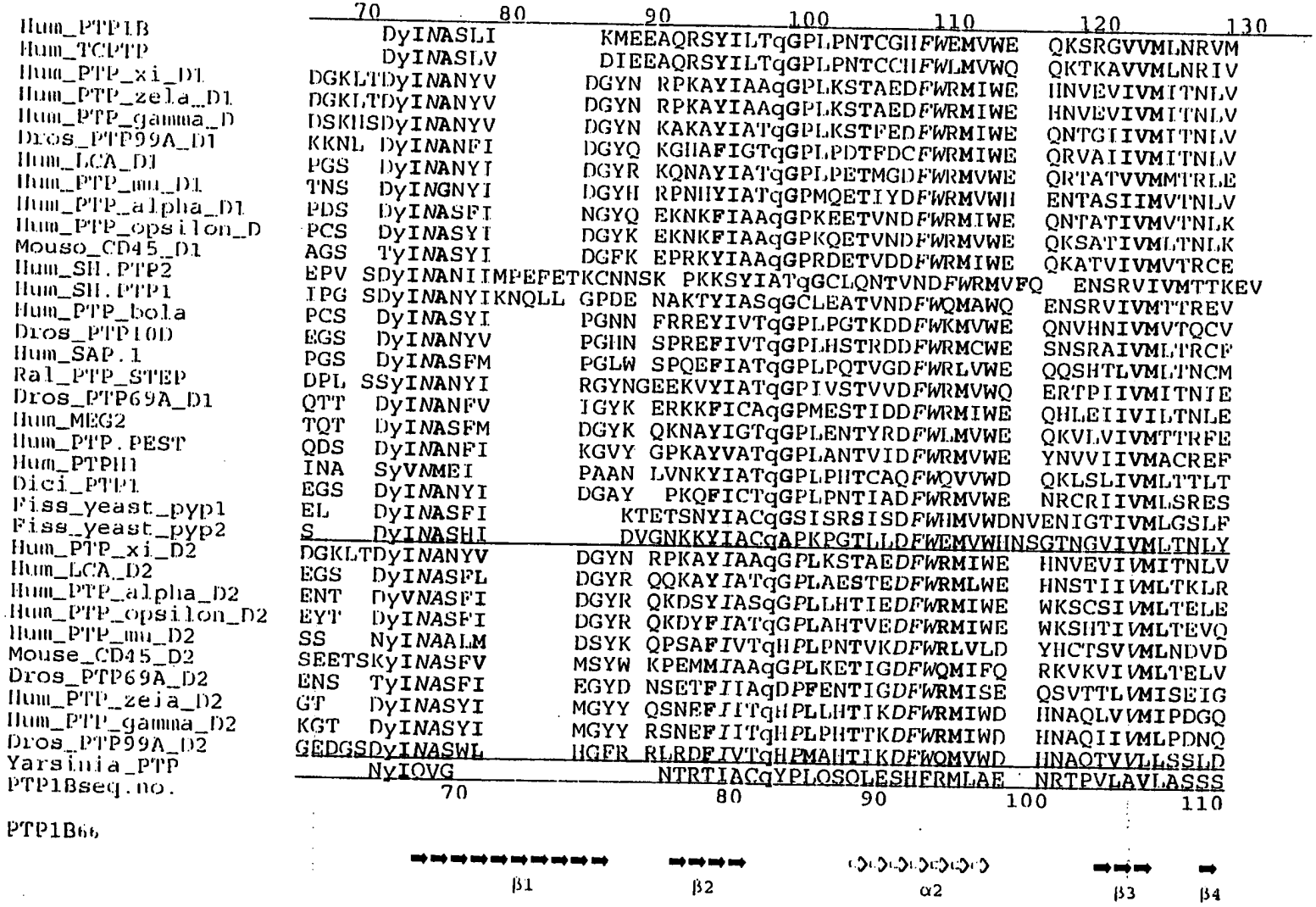
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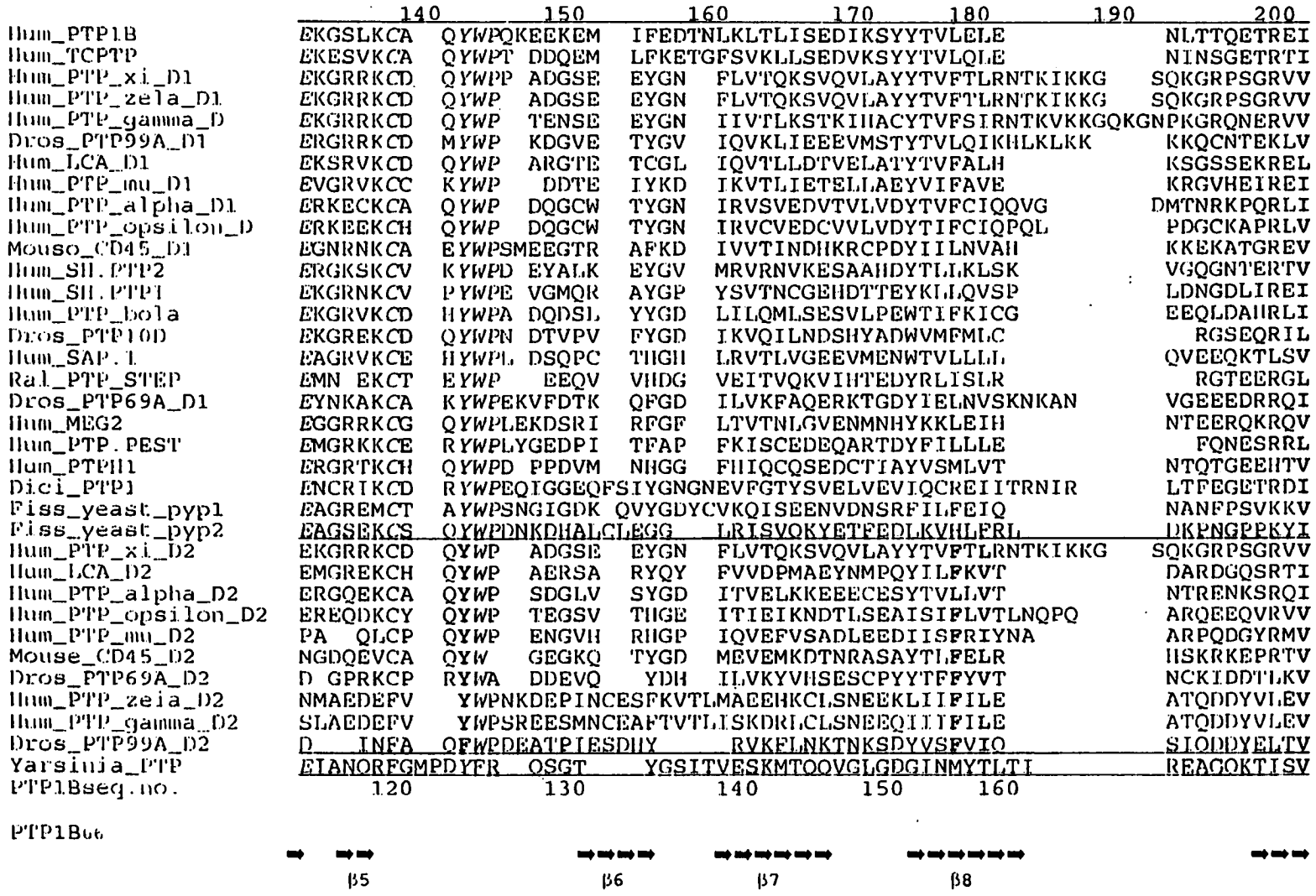
FIG. 1B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Hum_PTP_gamma_D EKGRRKCD QYWP TENSE EYGN IIVTLKSTKIHACYTVFSIRNTKVKKGQKGNPKGRQNERVV
Dros_PTP99A_D1 ERGRRKCD MYWP KDGVE TYGV IQVKLIEEEVMSTYTVLQIKHLKLKK KKQCNTEKLV
Hum_LCA_D1 EKSRVKCD QYWP ARGTE TCGL IQVTLLDTVELATYTVFALH KSGSSEKREL
Hum_PTP_mu_D1 EVGRVKCC KYWP DDTE IYKD IKVTLIETELLAEYVIFAVE KRGVHEIREI
Hum_PTP_alpha_D1 ERKECKCA QYWP DQGCW TYGN IRVSVEDVTVLVDYTVFCIQQVG DMTNRKPQRLI
Hum_PTP_opsilon_D ERKEEKCH QYWP DQGCW TYGN IRVCVEDCVVLVDYTIFCIQPQL PDGCKAPRLV
Mouse_CD45_D1 EGNRNKCA EYWPSMEEGTR AFKD IVVTINDHKRCPDYIILNVAH KKEKATGREV
Hum_SH.PTP2 ERGKSKCV KYWPD EYALK EYGV MRVRNVKESAAHDYTLLKLSK VGQGNTERTV
Hum_SH.PTP1 EKGRNKCV PYWPE VGMQR AYGP YSVTNCGEHDTTEYKLLQVSP LDNGDLIREI
Hum_PTP_bola EKGRVKCD HYWPA DQDSL YYGD LILQMLSESVLPEWTIFKICG EEQLDAIIRLI
Dros_PTP10D EKGREKCD QYWPN DTVPV FYGD IKVQILNDSHYADWVMFMLC RGSEQRIL
Hum_SAP.1 EAGRVKCE HYWPL DSQPC TIGH LRVTLVGEEVMENWTVLLLL QVEEQKTLSV
Ral_PTP_STEP EMN EKCT EYWP EEQV VHDG VEITVQKVIHTEDYRLISLR RGTEERGL
Dros_PTP69A_D1 EYNKAKCA KYWPEKVFDTK QFGD ILVKFAQERKTGDYIELNVSKNKAN VGEEEDRRQI
Hum_MEG2 EGGRRKCG QYWPLEKDSRI RFGF LTVTNLGVENMNHYKKLEIH NTEERQKRQV
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Hum_PTPH1 ERGRTKCH QYWPD PPDVM NHGG FIIQCQSEDCTIAYVSMLVT NTQTGEEHTV
Dici_PTP1 ENCRIKCD RYWPEQIGGEQFSIYGNGNEVFGTYSVELVEVIQCREIITRNIR LTFEGETRDI
Fiss_yeast_pyp1 EAGREMCT AYWPSNGIGDK QVYGDYCVKQISEENVDNSRFILFEIQ NANFPSVKKV
Fiss_yeast_pyp2 EAGSEKCS QYWPDNKDHALCLEGG LRISVQKYETFEDLKVHLFRL DKPNGPPKYI
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Hum_PTP_alpha_D2 ERGQEKCA QYWP SDGLV SYGD ITVELKKEEECESYTVLLVT NTRENKSRQI
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Hum_PTP_zeta_D2 NMAEDEFV YWPNKDEPINCESFKVTLMAEEHKCLSNEEKLIIFILE ATQDDYVLEV
Hum_PTP_gamma_D2 SLAEDEFV YWPSREESMNCEAFTVTLISKDRLCLSNEEQIIIFILE ATQDDYVLEV
Dros_PTP99A_D2 D INFA QFWPDEATPIESDHY RVKFLNKTNKSDYVSFVIQ SIQDDYELTV
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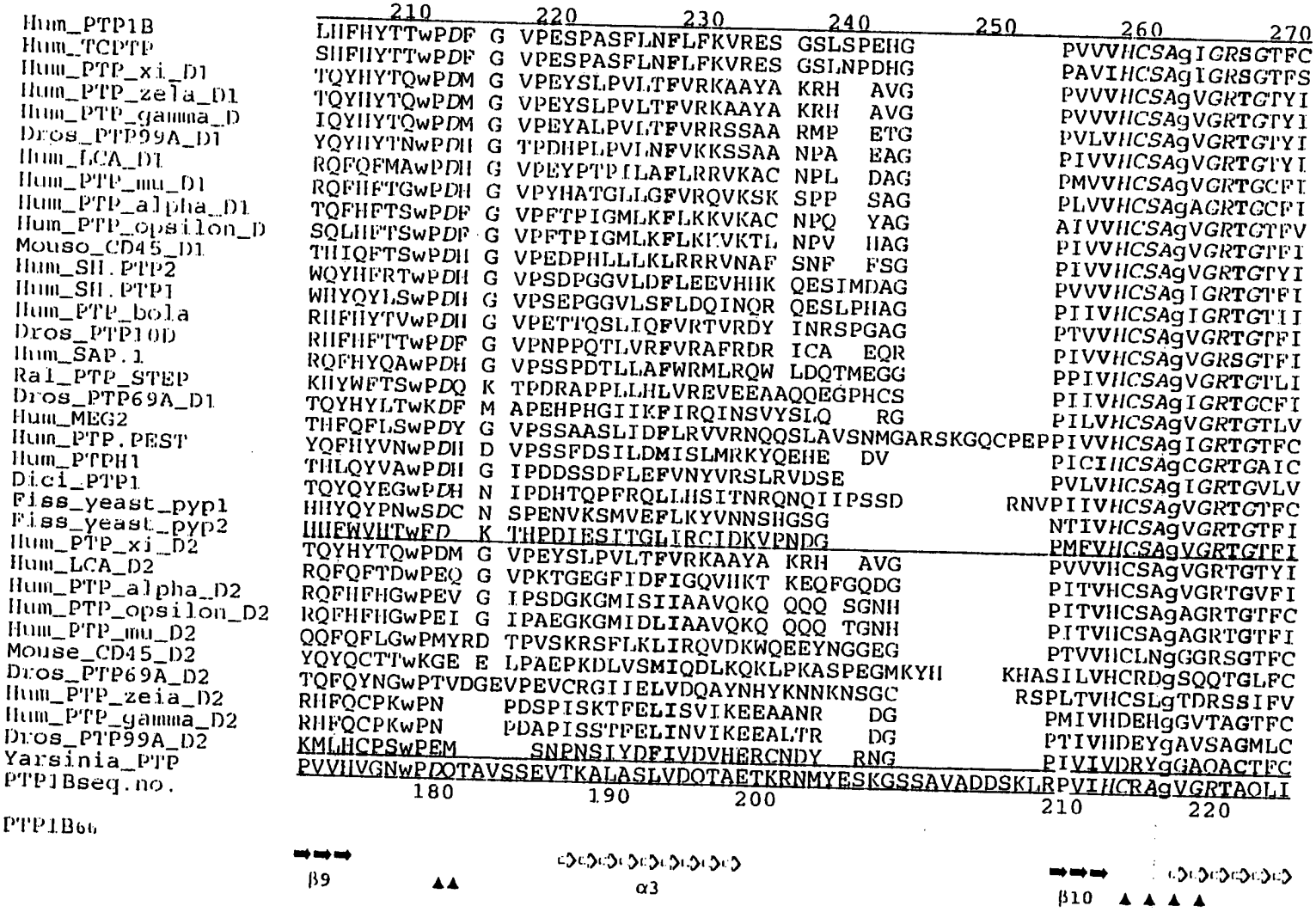
PTP1Bsec
β5 β6 β7 β8

FIG. 1C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Hum_PTP_alpha_D1
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Mouso_CD45_D1
Hum_SH.PTP2
Hum_SH.PTP1
Hum_PTP_bola
Dros_PTP10D
Hum_SAP.1
Ral_PTP_STEP
Dros_PTP69A_D1
Hum_MEG2
Hum_PTP.PEST
Hum_PTPH1
Dici_PTP1
Fiss_yeast_pyp1
Fiss_yeast_pyp2
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Hum_PTP_alpha_D2
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PTP1B66
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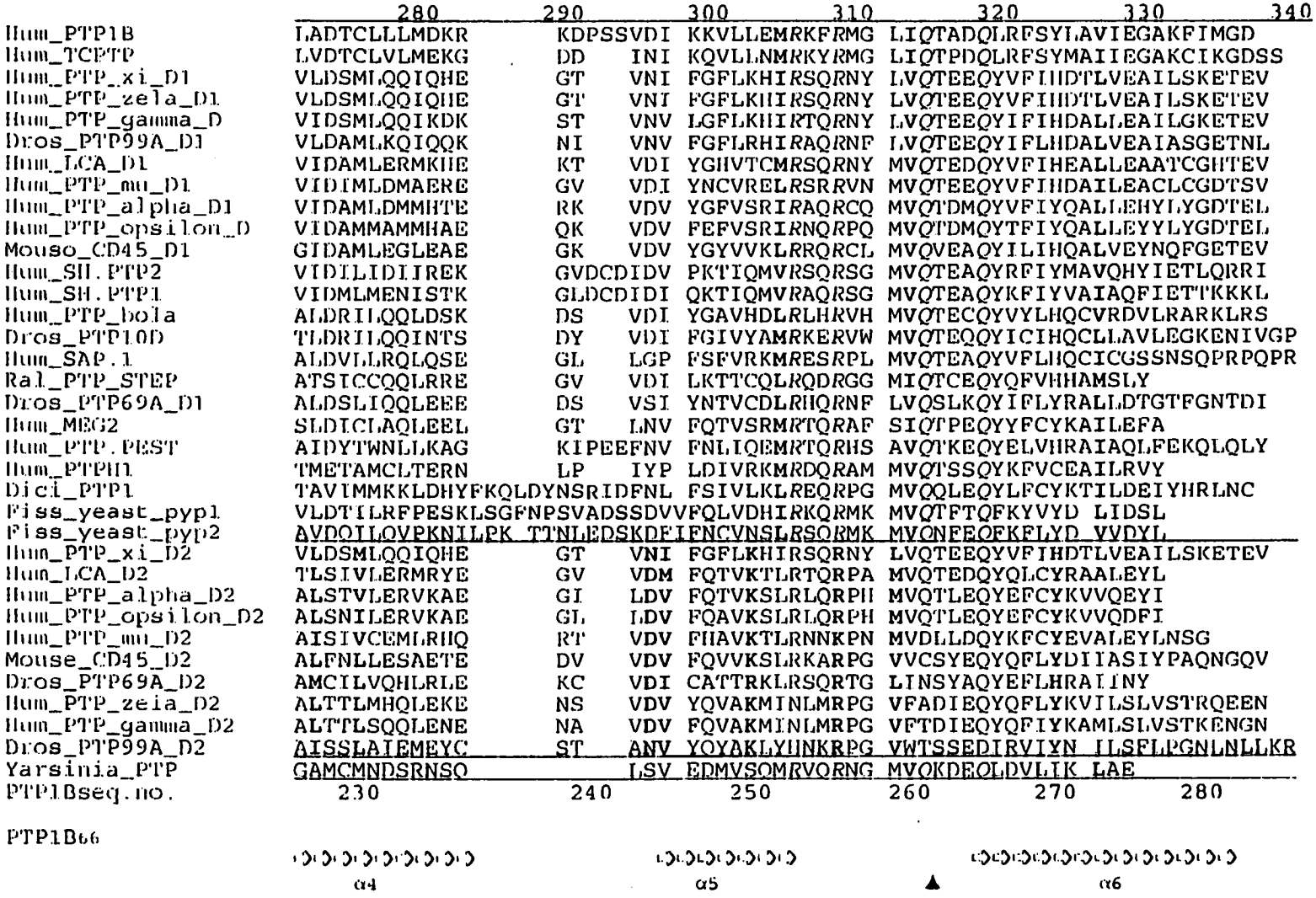
180 190 200 210 220
β9
α3
β10

FIG. 1D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Hum_PTP_zeta_D1 VLDSMLQQIQHE GT VNI FGFLKHIRSQRNY LVQTEEQYVFIHDTLVEAILSKETEV
Hum_PTP_gamma_D VIDSMLQQIKDK ST VNV LGFLKHIRTQRNY LVQTEEQYIFIHDALLEAILGKETEV
Dros_PTP99A_D1 VLDAMLKQIQQK NI VNV FGFLRHIRAQRNF LVQTEEQYIFLHDALVEAIASGETNL
Hum_LCA_D1 VIDAMLERMKHE KT VDI YGHVTCMRSQRNY MVQTEDQYVFIHEALLEAATCGHTEV
Hum_PTP_mu_D1 VIDIMLDMAERE GV VDI YNCVRELRSRRVN MVQTEEQYVFIHDAILEACLCGDTSV
Hum_PTP_alpha_D1 VIDAMLDMMHTE RK VDV YGFVSRIRAQRCQ MVQTDMQYVFIYQALLEHYLYGDTEL
Hum_PTP_epsilon_D VIDAMMAMMHAE QK VDV FEFVSRIRNQRPQ MVQTDMQYTFIYQALLEYYLYGDTEL
Mouso_CD45_D1 GIDAMLEGLEAE GK VDV YGYVVKLRRQRCL MVQVEAQYILIHQALVEYNQFGETEV
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Hum_SAP.1 ALDVLLRQLQSE GL LGP FSFVRKMRESRPL MVQTEAQYVFLHQCICGSSNSQPRPQPR
Ral_PTP_STEP ATSICCQQLRRE GV VDI LKTTCQLRQDRGG MIQTCEQYQFVHHAMSLY
Dros_PTP69A_D1 ALDSLIQQLEEE DS VSI YNTVCDLRHQRNF LVQSLKQYIFLYRALLDTGTFGNTDI
Hum_MEG2 SLDICLAQLEEL GT LNV FQTVSRMRTQRAF SIQTPEQYYFCYKAILEFA
Hum_PTP.PEST AIDYTWNLLKAG KIPEEFNV FNLIQEMRTQRHS AVQTKEQYELVHRAIAQLFEKQLQLY
Hum_PTPH1 TMETAMCLTERN LP IYP LDIVRKMRDQRAM MVQTSSQYKFVCEAILRVY
Dici_PTP1 TAVIMMKKLDHYFKQLDYNSRIDFNL FSIVLKLREQRPG MVQQLEQYLFCYKTILDEIYHRLNC
Fiss_yeast_pyp1 VLDTILRFPESKLSGFNPSVADSSDVVFQLVDHIRKQRMK MVQTFTQFKYVYD LIDSL
Fiss_yeast_pyp2 AVDQILQVPKNILPK TTNLEDSKDFIFNCVNSLRSQRMK MVQNFEQFKFLYD VVDYL
Hum_PTP_xi_D2 VLDSMLQQIQHE GT VNI FGFLKHIRSQRNY LVQTEEQYVFIHDTLVEAILSKETEV
Hum_LCA_D2 TLSIVLERMRYE GV VDM FQTVKTLRTQRPA MVQTEDQYQLCYRAALEYL
Hum_PTP_alpha_D2 ALSTVLERVKAE GI LDV FQTVKSLRLQRPH MVQTLEQYEFCYKVVQEYI
Hum_PTP_epsilon_D2 ALSNILERVKAE GI LDV FQAVKSLRLQRPH MVQTLEQYEFCYKVVQDFI
Hum_PTP_mu_D2 AISIVCEMLRHQ RT VDV FHAVKTLRNNKPN MVDLLDQYKFCYEVALEYLNSG
Mouse_CD45_D2 ALFNLLESAETE DV VDV FQVVKSLRKARPG VVCSYEQYQFLYDIIASIYPAQNGQV
Dros_PTP69A_D2 AMCILVQHLRLE KC VDI CATTRKLRSQRTG LINSYAQYEFLHRAILNY
Hum_PTP_zeta_D2 ALTTLMHQLEKE NS VDV YQVAKMINLMRPG VFADIEQYQFLYKVILSLVSTRQEEN
Hum_PTP_gamma_D2 ALTTLSQQLENE NA VDV FQVAKMINLMRPG VFTDIEQYQFIYKAMLSLVSTKENGN
Dros_PTP99A_D2 AISSLAIEMEYC ST ANV YQYAKLYINKRPG VWTSSEDIRVIYN ILSFLPGNLNLLKR
Yarsinia_PTP GAMCMNDSRNSQ LSV EDMVSQMRVQRNG MVQKDEQLDVLIK LAE
PTP1Bseq.no. 230 240 250 260 270 280
PTP1B66
α4 α5 α6

FIG. 1E

Vmax and Km of 37kDa-PTP1B Mutants Toward RCML

Enzyme	Vmax (nmol/min/mg)	Km (nM)	Kcat (min^{-1})
wild type	60200	102	2244
Tyr 46 → S	4120	1700	154
→ L	4160	1700	155
Glu 115 → A	5700	45	212
→ D	5900	20	220
Lys 116 → A	68600	150	2557
Lys 120 → A	19000	80	708
Asp 181 → A	0.61	≤126	0.023
→ E	97	10	3.6
His 214 → A	700	20	26
Cys 215 → S	0.026		0.00097
Arg 221 → K	11	80	0.41
→ M	3.3	1060	0.12
Gln 262 → A	720	9	27

FIG. 2

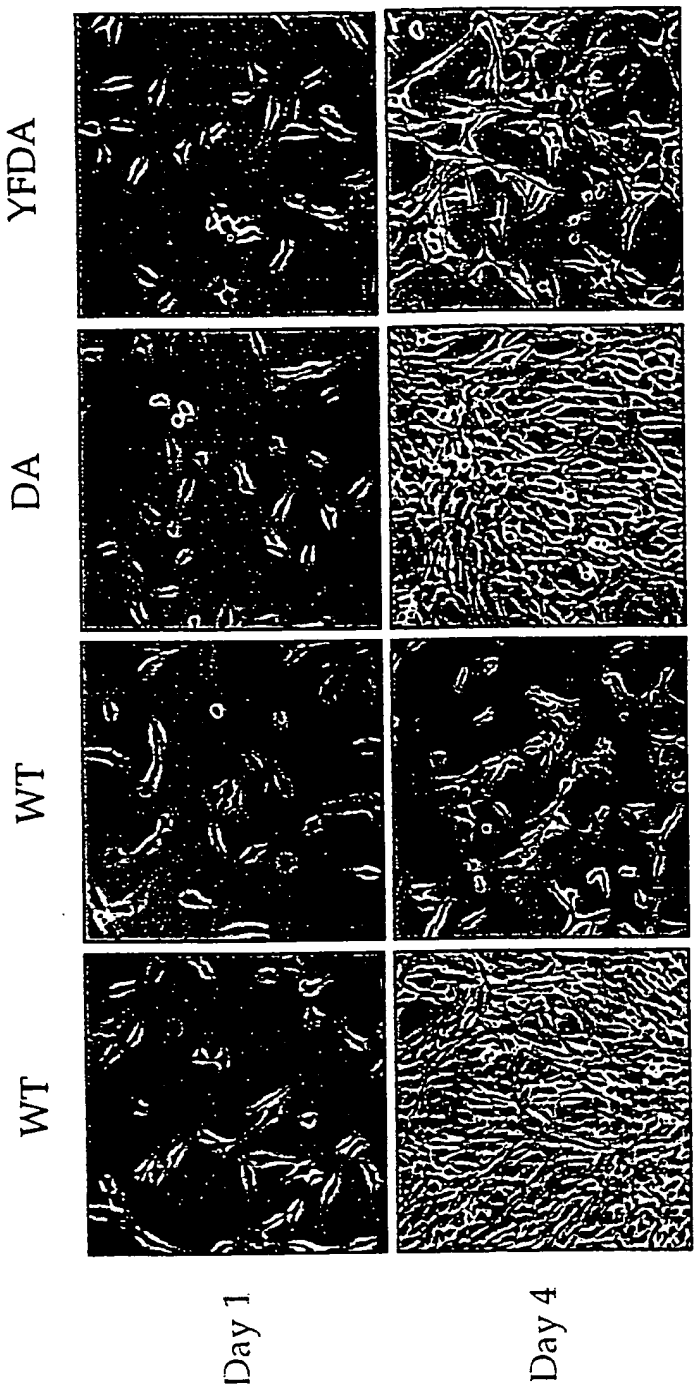
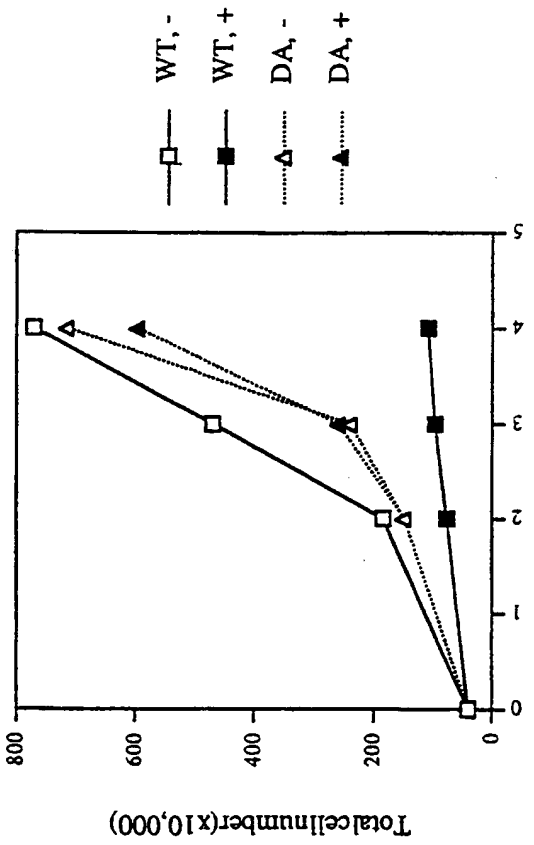


FIG. 3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DA, -
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400
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0
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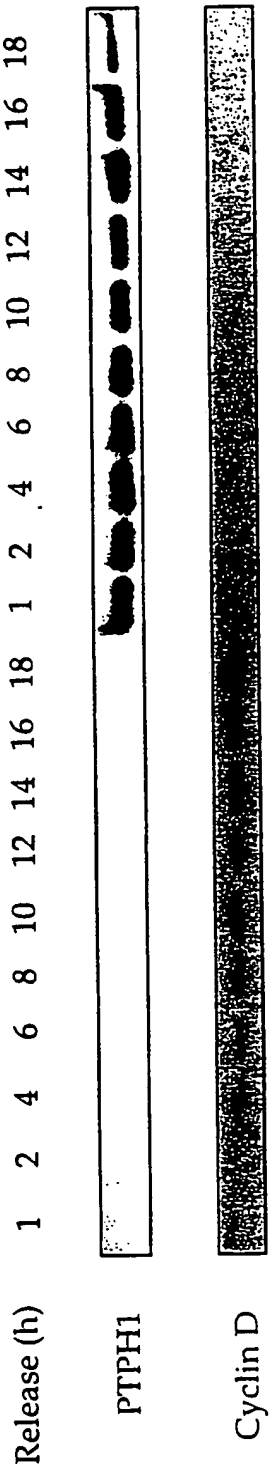
1
2
3
4
5
Day

FIG. 4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PTPH1
Cyclin D
-
+
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FIG. 5

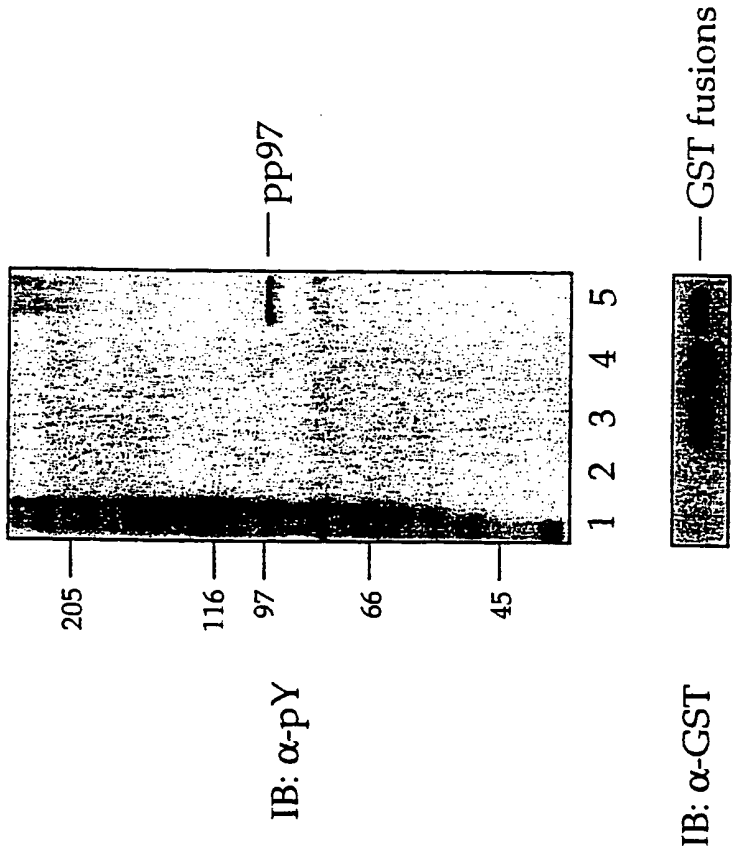

FIG. 6

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DTVEGITGNLFEVYLK**PYFLEAYRPIR**KGDIFLVRGGMRAVEFKVVETDPSPYCIVAPDT
VIHCEGEPIK**REDEEESLNE**VGYDDVGGCRKQLAQIKEMVELPLRHPALFK**AIGVKPPRG**
ILLYGPPGTGKTLIARAVANETGAFFFLINGPEIMSKLAGESESNLRKAFEEAEKNAPAI
IFIDELDAIAPKREKTHGEVERRIVSQLLTLMDGLKQRAHVIVMAATNRPNSIDPALRRF
GRFDREVDIGIPDATGRLEILQIHTKNMKLADDVDLEQVANETHGHVGADLAALCSEAAL
QAIRKKMDLIDLEDETIDAEVMNSLAVTMDDFRWALSQSNPSALRETVVEVPQVTWEDIG
GLEDVK**RELQELVQYPVEHPD**KFLKFGMTPSKGVLFYGPPGCGKTLLAKAIANECQANFI
SIKGPELLTMWFGESEANVREIFDKARQAAPCVLFFDELDSIAKARGGNIGDGGGAADRV
INQILTEMDGMSTKKNVFIIGATNRPDIIDPAILRPGRLDQLIYIPLPDEKSRVAILKAN
LRKSPVAK**DVDLEFLAKM**TNGFSGADLTEICQRACKLAIRESIESEIRRERERQTNPSAM
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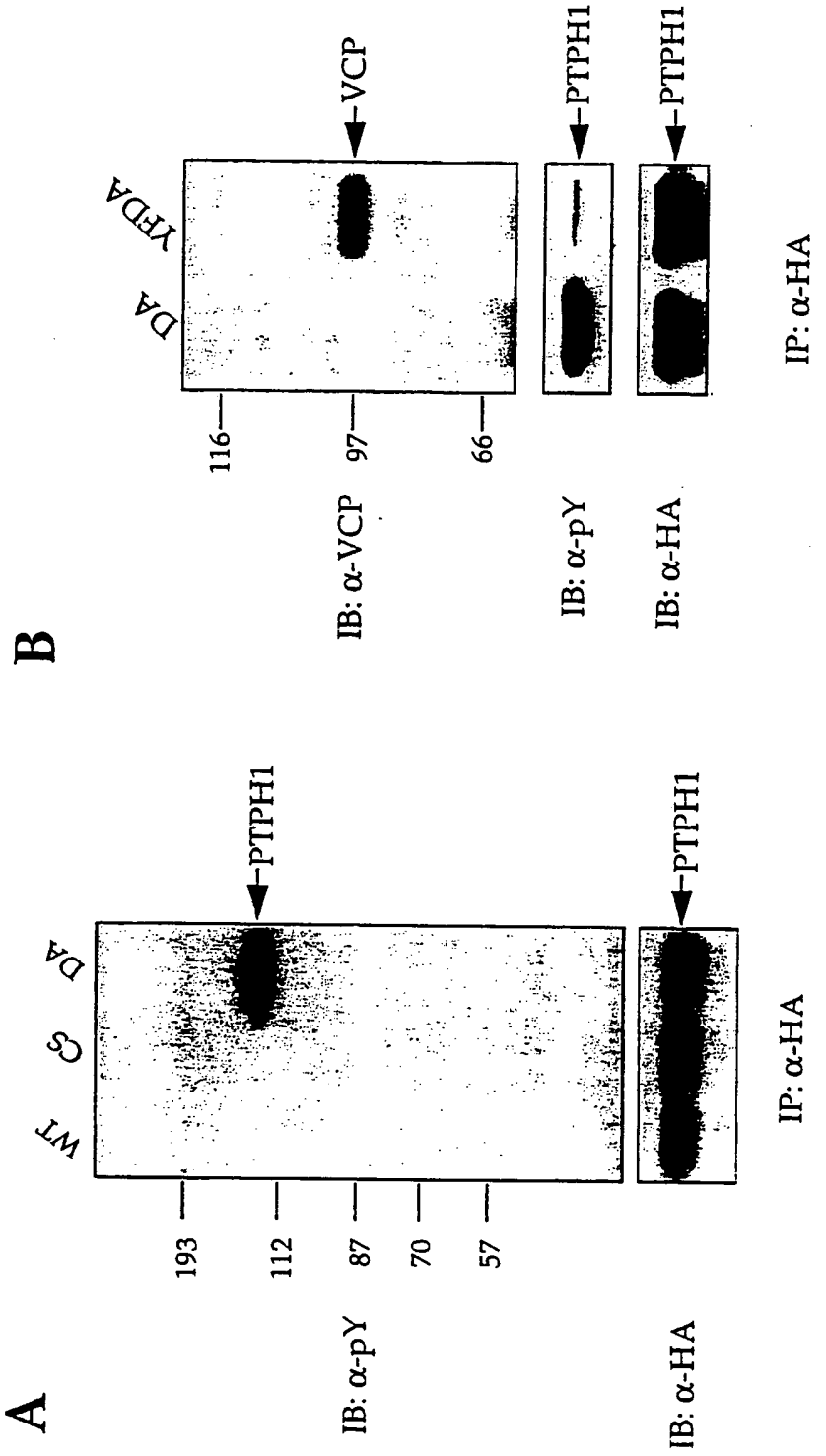
FIG. 7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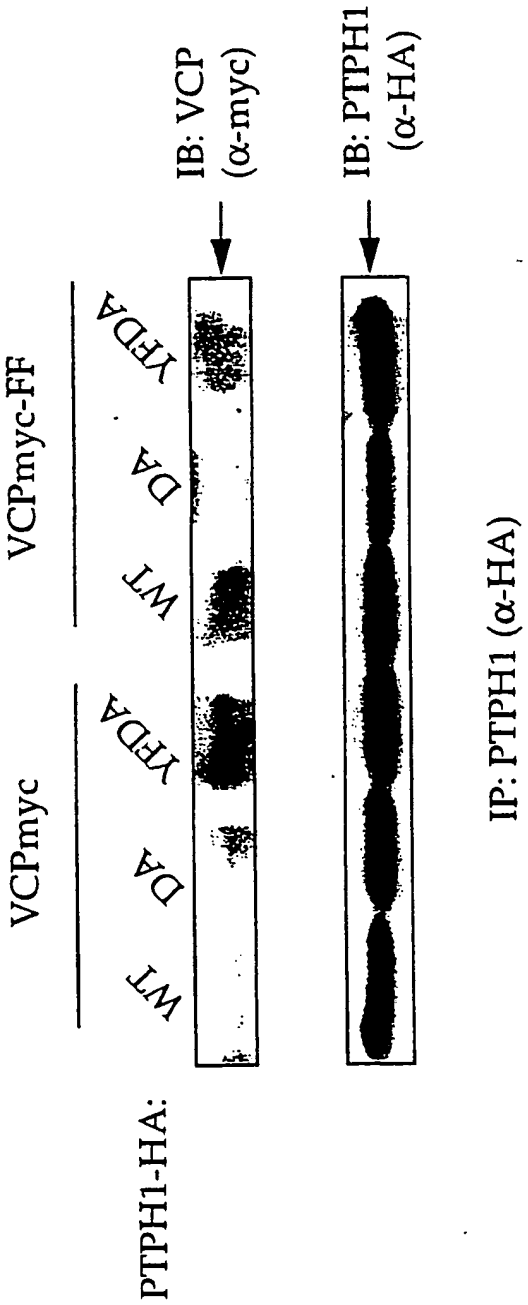
IP: α-HA

FIG. 8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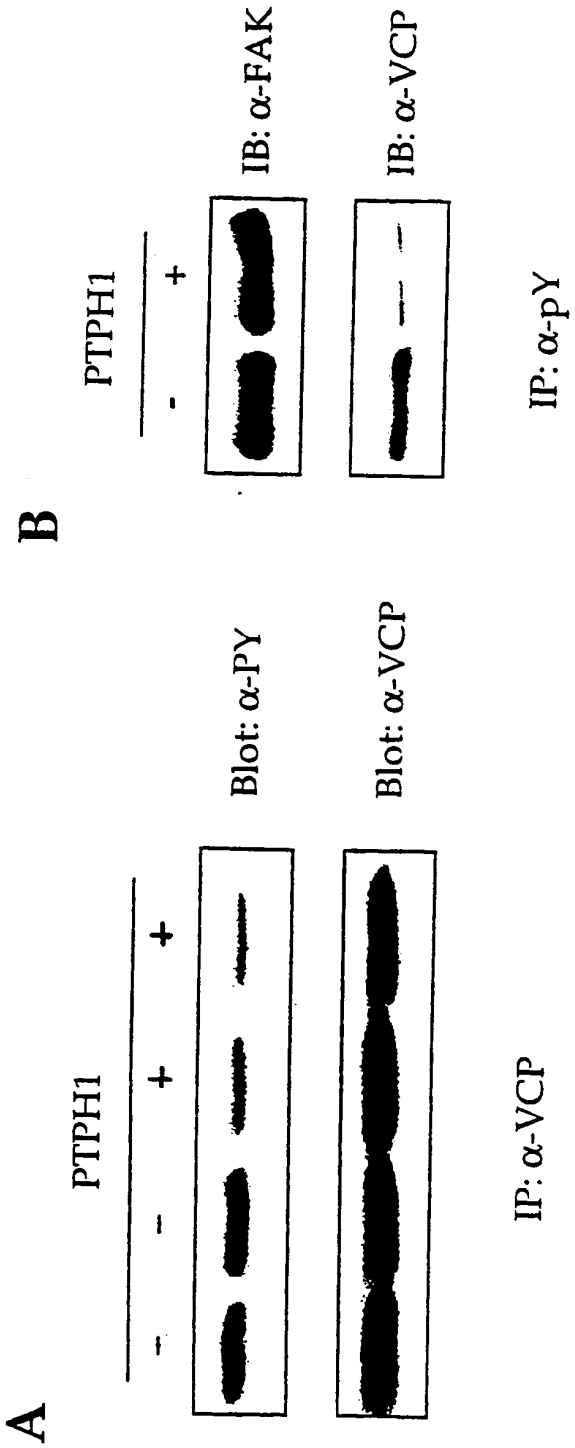
IP: PTPH1 (α-HA)

FIG. 9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- +
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IP: α-pY

FIG. 10

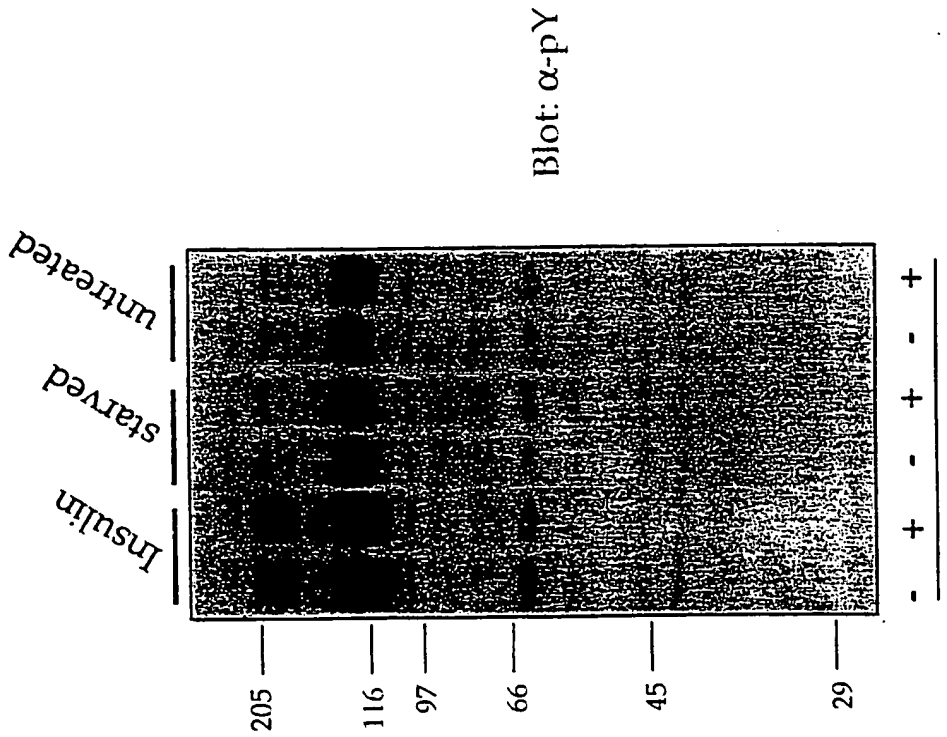

FIG. 11

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 00/14211

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/52 C12N9/16 C12N15/62 A61K38/00 C12N15/11
G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
STRAND, BIOSIS, EMBASE, PAJ, WPI Data, CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J.W. ECKSTEIN ET AL.: "Identification of an essential acidic residue in Cdc25 protein phosphatase and a general three-dimensional model for a core region in protein phosphatases." PROTEIN SCIENCE, vol. 5, no. 1, January 1996 (1996-01), pages 5-12, XP002051424 figure 4 --- -/--	1-54

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 October 2000	09/10/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Hix, R

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/14211

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	A.J. FLINT ET AL.: "Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases" PROC. NATL. ACAD. SCI. USA, vol. 94, March 1997 (1997-03), pages 1680-1685, XP002051429 cited in the application the whole document	1-54
Y	A.J. GARTON ET AL.: "Identification of p130cas as a substrate for teh cytosolic protein tyrosine phosphatase PTP-PEST." MOLECULAR AND CELLULAR BIOLOGY, vol. 16, no. 11, November 1996 (1996-11), pages 6408-6418, XP002051430 the whole document	1-54
Y	B.L. SEELY ET AL.: "Protein tyrosine phosphatase 1B interacts with the activated insulin receptor." DIABETES, vol. 45, no. 10, October 1996 (1996-10), pages 1379-1385, XP002051432 the whole document	1-54
Y	LAMONTAGNE K.R. JR. ET AL: "Protein tyrosine phosphatase PTP1B suppresses p210 bcr-abl-induced transformation of rat-1 fibroblasts and promotes differentiation of K562 cells." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (24 NOV 1998) 95/24 (14094-14099). , XP000861906 the whole document	1-54
Y	TIGANIS T. ET AL: "Epidermal growth factor receptor and the adaptor protein p52(Shc) are specific substrates of T-cell protein tyrosine phosphatase." MOLECULAR AND CELLULAR BIOLOGY, (1998) 18/3 (1622-1634). , XP000861959 cited in the application the whole document	1-54
Y	WO 98 04712 A (COLD SPRING HARBOR LAB) 5 February 1998 (1998-02-05) cited in the application the whole document	1-54

-/--

1

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/14211

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZHANG S.-H. ET AL: "Identification of the cell cycle regulator VCP (p97/CDC48) as a substrate of the band 4.1-related protein - tyrosine phosphatase PTPH1." JOURNAL OF BIOLOGICAL CHEMISTRY, (18 JUN 1999) 274/25 (17806-17812). , XP000938707 the whole document	1-54
A	Q. YANG ET AL.: "Cloning and expression of PTP-PEST." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 9, 25 March 1993 (1993-03-25), pages 6622-6628, XP002034265 cited in the application figure 2	
A	F. LIU ET AL.: "Direct binding of a proline-rich region of protein tyrosine phosphatase 1B to the Src Homology 3 Domain of p130 cas" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 49, 6 December 1996 (1996-12-06), pages 31290-31295, XP002051433 the whole document	
A	MYERS M.P. ET AL: "The lipid phosphatase activity of PTEN is critical for its tumor supresso function." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (10 NOV 1998) 95/23 (13513-13518). , XP000861905	

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INTERNATIONAL SEARCH REPORT

formation on patent family members

Interr nal Application No
PCT/US 00/14211

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9804712 A	05-02-1998	US 5912138 A	15-06-1999
		AU 5939598 A	16-02-1999
		CA 2262440 A	05-02-1998
		EP 0918867 A	02-06-1999
		US 5951979 A	14-09-1999